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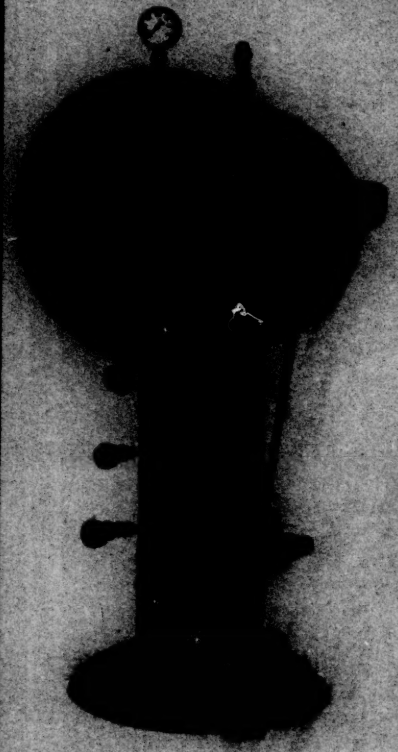

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THE HYDROGEN ION CONCENTRATION OF THE CONTENTS OF THE SMALL INTESTINE.

By J. F. McCLENDON, A. SHEDLOV, and BEN KARPMAN.

(From the Physiological Laboratory of the University of Minnesota Medical School, Minneapolis.)

(Received for publication, February 19, 1918.)

Although the prevailing idea is that the reaction of the small intestine is alkaline (Auerbach and Pick), it was shown by the senior author that the duodenal content of infants is distinctly acid (pH = 3.1), and by McClendon, Shedlov, and Thomson that the content of the ileum of pups, whether fed on milk or on solid food, is slightly acid (pH = 5.7 to 6.34), and that the duodenum of the adult dog has a similar hydrogen ion concentration. In the present paper we describe the determinations showing that the small intestine of the dog is slightly acid throughout its entire length.

Owing to the very rapid absorption from the dog's intestine it is sometimes difficult to get fluid out of the lower parts without the administration of some laxative, and in the following experiments magnesium sulfate was sometimes given, as indicated in the table below. The dogs were fed on a mixed diet of cooked food, and the abdominal cavity was opened under ether anesthesia, and samples of the intestinal contents were drawn directly into hydrogen electrodes, described by McClendon and Magoon, through punctures in the intestinal wall. The hydrogen ion concentration was determined with the aid of a Leeds and Northrup potentiometer and galvanometer. In the following table the dogs were numbered in the order received (first column), the number of hours after feeding at which the samples were taken is indicated in the second column, and the pH($= -\log H_+$) in the third column. Under "Remarks" is given the part of the intestine from which the sample was taken, and magnesium sulfate is indicated in case it was administered.

Dog No.	Hour.	pH	Remarks.
1	$2\frac{3}{4}$	6.3	First part of ileum.
		6.3	" " " " (duplicate).
2	$3\frac{1}{2}$	5.6	" " " "
		5.7	Middle " " "
3	4	5.7	" " " " $MgSO_4$.
		5.6	" " " " "
4	$4\frac{1}{2}$	6.4	First " " " "
		6.6	Last " " " "

The pH of the stomach was determined in one case during the early part of digestion and found to be 3.55, but this must decrease during digestion since the pH of the gastric juice is about 1. Boldyreff has shown that the pancreatic juice is chiefly instrumental in neutralizing the gastric juice, and, according to Foà, dog's bile is slightly acid. If we grant that there is more than enough sodium bicarbonate in the pancreatic juice to neutralize the gastric juice, the reaction of the small intestine would probably be acid due to the liberation of CO_2 by the gastric juice, the presence of acid albuminate, and the formation of amino-acids. This acidity would be increased if acid-forming bacteria were present. Since Bulgarian bacilli and other acid-forming bacteria are administered in order to prevent abnormal intestinal fermentation, it seems probable that these maintain a distinctly acid reaction in the intestine. Michaelis and Clark and Lubs have shown that bacterial inhibition occurs at about pH 5. There seems to be some uncertainty, however, as to the general application of the conclusions of Michaelis.

Although it is often stated that an alkaline reaction is necessary for pancreatic proteolysis, this seems to be improbable. At any rate, intestinal digestion appears to proceed rapidly in the dog.

BIBLIOGRAPHY.

Auerbach, F., and Pick, H., *Arb. k. Gsndsamte.*, 1912, xliii, 155.
Clark, W. M., and Lubs, H. A., *J. Infect. Dis.*, 1915, xvii, 109, 160.
Foà, C., *Arch. fisiol.*, 1905–06, iii, 390.
McClendon, J. F., *Am. J. Physiol.*, 1915, xxxviii, 191.
McClendon, J. F., and Magoon, C. A., *J. Biol. Chem.*, 1916, xxv, 669.
McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, xxxi, 269.
Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 112.

THE PLACENTAL TRANSMISSION OF CREATININE AND CREATINE.

By ANDREW HUNTER and WALTER R. CAMPBELL.

(*From the Department of Pathological Chemistry, University of Toronto.*)

(Received for publication, February 8, 1918.)

Some time ago, in a preliminary communication[1] dealing with various aspects of the distribution of creatinine and creatine in blood, we reported that the concentration of each of these substances was nearly the same, at parturition, in the placental as in the maternal plasma; and we concluded that there takes place between the mother and the fetus a ready interchange of both. If we have hesitated hitherto to publish any fuller account of these observations, it is chiefly because of the necessity under which we found ourselves of first testing rather thoroughly the validity of the analytical methods used.[2] In the meantime our results have been confirmed by the independent observations of Plass.[3,4]

[1] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii. The work upon the placental transmission of creatinine and creatine, of which this communication contained of course no more than an abstract, constituted in our original intention one part only of an extended program, then already under way, for the study of placental interchange in general. Until we first made it public, we were not aware that an investigation of similar scope had been for some time in progress in another laboratory, and that results concerning the placental transmission of total non-protein nitrogen and urea had already been reported by Slemons and Morriss.[5] When these circumstances were brought to our notice we felt bound to restrict ourselves, in the further prosecution of our plan, to the cases of creatinine and creatine, to which Slemons and his coworkers had not yet turned their attention. We are indebted to Dr. Slemons for the ready courtesy with which he left free to us this corner of the field in which he was engaged. It may be remarked here that such unpublished data as we had collected upon other constituents of the blood are in complete harmony with the reported conclusions of the Yale laboratory.

[2] Hunter and Campbell, *J. Biol. Chem.*, 1917, xxxii, 195.

[3] Plass, E. D., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137.

[4] Plass, *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 297.

For this reason we have deemed it unnecessary, since completing our critical study of the methods, to supplement our original data by more than a few additional determinations. The point of view from which we approached the subject has been briefly indicated in previous communications.[1,5] It is identical, moreover, with that taken, in reference to the general problem of placental exchange, by Plass and by Slemons and Morriss.[6] As these authors have discussed it with sufficient fullness and with ample reference to the earlier literature, any further elaboration of it here would be superfluous. The main object of the present paper is simply to record in detail the analytical results we have collected, and to consider, in the light of our lately formed estimate of their reliability, how far they justify our original conclusion.

Subjects and Methods.

The cases selected were full term deliveries,[7] complicated in two instances only (Nos. 16 and 18) by the application of forceps. The only abnormality noted in the course of pregnancy was a mild albuminuria in Case 8. In three cases (Nos. 9, 10, and 18) labor was assisted by the administration of pituitrin. In all, light chloroform-ether anesthesia was induced at the close of the second stage. As soon as the umbilical cord was cut, a sample of blood was taken from the free outflow of its placental end, and as nearly as possible at the same time a specimen was secured from the arm vein of the mother. Coagulation was prevented in each case by the addition of about one-fiftieth volume of 20 per cent potassium oxalate. Upon each sample, as secured, we determined the creatinine and creatine of whole blood and plasma, as well as, with the object of calculating the corpuscular concentration of these substances, the relative plasma volume. The details of technique adopted were those described in a former

[5] Hunter and Campbell, *J. Biol. Chem.*, 1918, xxxiii, 169.

[6] Slemons, J. M., and Morriss, W. H., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 343.

[7] All were inmates of the obstetrical wards of the Toronto General Hospital. For permission to utilize them in this study we are indebted to Doctors B. P. Watson and K. C. McIlwraith, whose courtesy we wish to acknowledge.

paper.[5] Except in Cases 1 and 4 the blood was laked before the addition of picric acid.

Results.

The results are recorded in the accompanying table.[8] This does not reproduce any selected group, but includes every analysis made in this particular series. The order of arrangement

No.	Subject.	Age.	Para.	Duration of labor.		Creatinine per 100 cc.				Creatine (as creatinine) per 100 cc.				Relative plasma volume.	
						Whole blood.		Plasma.		Whole blood.		Plasma.			
				First stage.	Second stage.	M*	F†	M	F	M	F	M	F	M	F
		yrs.		*hrs.*	*hrs.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1	S. G.	37	XIV	7	1	1.1	1.5	0.74	0.87	6.2	5.7	1.8	1.8	61	71
2	A. D.	23	III	3¾	¼	1.1	1.4	0.75	0.88	8.8	10.5	1.3	1.9	62	49
3	P. A.	23	II	2½	½	2.8	3.4	0.82	0.96	5.5	7.9	1.9	2.1	62	42
4	C. S.	30	II	8	1½	1.4	1.9	0.87	0.75	6.2	6.4	1.3	1.8	73	69
5	F. G.	23	I	19¾	2¼	2.3	3.5	0.89	1.14	7.7	6.9	2.3	2.4	62	47
6	I. M.	21	I	9½	2⅓	2.1	2.3	0.92	0.89	8.6	9.4	1.6	2.5	65	53
7	A. S.	26	IV	13½	⅙	1.8	3.3	0.92	0.89	8.8	8.6	2.3	2.3	65	52
8	M. B.	25	III	38	¼	2.1	2.0	0.97	1.21	5.8	11.0	1.4	1.9	54	47
9	E. H.	29	II	9¾	⅔	2.3	3.8	1.00	1.16	8.2	8.3	2.0	2.3	61	56
10	A. C.	25	I	51½	2	1.9	3.0	1.02	1.65	7.7	7.7	3.9	3.4	59	45
11	M. O.	28	I	13¼	?	2.5	3.0	1.02	1.02	8.8	11.3	1.7	2.3	69	49
12	N. P.	17	I	7¼	¾	2.4	2.3	1.16	0.80	5.6	7.5	3.1	2.5	58	62
13	V. S.	18	I	23	2¾	4.0	2.4	1.26	1.40	2.8	5.6	1.3	1.7	58	59
14	E. A.	22	I	11	2¾	2.2	2.7	1.37	1.62	9.3	10.5	5.3	2.3	57	43
15	E. T.	19	I	2⅔	1	3.2	3.5	1.51	1.30	2.6	4.2	0.9	1.4	58	57
16	M. F.	21	I	23	11	3.1	2.9	1.59	1.59	7.9	13.7	2.2	2.0	56	49
17	M. M.	?	?	?	?	2.5	3.0	1.77	1.71	—	—	—	—	—	—
18	B. H.	20	I	37½	2½	—	—	2.20	1.95	—	—	1.3	1.8	68	54
Average						2.3	2.7	1.15	1.21	6.9	8.5	2.09	2.14	62	53
" corrected										3.45	4.25	0.52	0.54		

* M = Maternal. †F = Fetal.

[8] They are taken, for the most part, from a thesis submitted by Walter R. Campbell, in partial fulfillment of the requirements for the degree of M.D., University of Toronto, 1917.

within the table is determined by the plasma creatinine of the maternal blood. The corrections introduced in the averages are made on the basis of considerations developed in earlier communications.[2,5]

DISCUSSION.

Placental Transmission of Creatinine.

For reasons that are obvious, and upon which others as well as ourselves have already sufficiently insisted,[3,5] it is the plasma concentrations alone which have a direct bearing upon the problem of placental exchange. It is therefore fortunate that, although with whole blood the colorimetric method of creatinine determination is subject to a considerable and variable error, one may accept as substantially correct the results of its application to plasma.[2,9] A study of such results, as they are recorded in the ninth and tenth columns of the table, reveals a general correspondence between the concentration of creatinine in the maternal and that in the fetal plasma. This correspondence is just as close at the upper end of the range illustrated as at the lower. In five instances out of the total eighteen it amounts to absolute or practical identity. In twelve the difference existing, if not negligible, is relatively unimportant. One case only (No. 10) exhibits a really conspicuous divergence.[10] The averages for the series fall closely together, and but for the inclusion of the aberrant, and not impossibly erroneous, values of Case 10, they would have been closer still. There can be little doubt, therefore, that the structures which separate the fetal and maternal plasmas are freely permeable to creatinine, and that the passage of that substance from one circulation to the other is regulated by the ordinary laws of diffusion. Our earlier report is fully substantiated in this respect.

Whether our figures will bear any closer analysis may well be

[9] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

[10] It was a very prolonged dry labor during which pituitrin was administered. The possibility is suggested that these conditions led to some obstruction of the fetal circulation, and consequent accumulation of creatinine in the fetal blood; but it is equally likely that we are dealing with an accidental error of analysis. The amount of material unfortunately was too small to permit a duplicate determination.

doubted. We are tempted, nevertheless, to remark upon the fact that whereas there are only four cases (Nos. 4, 12, 15, and 18) in which the maternal concentration is distinctly higher than the fetal, there are as many as nine in which the reverse is the case. This may be no more than an accident, and yet it is conceivably of real significance. Creatinine is an excretory product of which the final disposition, even for the fetal moiety, is escape through the maternal kidney. While therefore any sudden accumulation within the maternal organism might temporarily favor its transference from mother to fetus, the main current of its diffusion must set in the opposite direction. It would therefore be not at all surprising if there existed, as seems at least to be indicated in our data, a preponderating tendency towards the establishment of a concentration gradient falling from fetus to mother. We hesitate to appeal, in further support of this conception, to the slight excess in favor of the fetal plasma which is presented in our averages; for that depends mainly, as we have stated, upon the doubtful result of Case 10. We may, however, point out that a reversal of the theoretically normal gradient does really appear to be associated as a rule with an unusual accumulation in the mother; for of the four cases which exemplify that condition one only has a maternal creatinine lower than 1.16, and 1.16, according to data already published,[5] is at the upper limit of normality for the closing weeks of pregnancy.[11]

Creatinine of Maternal and Fetal Whole Blood.

It has been made probable[5, 9] that the true creatinine content of whole blood is practically the same as that of its plasma. What has been said in the preceding paragraphs of the latter is therefore applicable at once to the former, and it is hardly necessary to make separate reference to the figures for whole blood yielded by direct analysis. To the extent to which these differ from the figures for plasma they are, we believe, in error. It may be

[11] Some evidence that these relations are not merely the result of chance is afforded by a scrutiny of the results of Plass.[3] Among the fourteen results for plasma creatinine which he reports (Tables III and IV) there are five which show a slight excess (of 0.05 or more) on the fetal side, as against three exhibiting the reverse condition; and in each of the latter the maternal creatinine is greater than 1.2.

remarked that, if this is granted, the results as found (columns seven and eight) not only give additional force to our suspicion that the error is especially conspicuous in the blood of pregnant women[2] but suggest that in fetal blood, perhaps because of its high content of corpuscles, it is even more so. Many of the observed results for whole blood would, taken by themselves, indicate an accumulation of creatinine, which the corresponding result for plasma shows to be wholly imaginary. Due caution therefore is to be exercised in interpreting the data of whole blood creatinine determinations in pregnant women or new-born infants.

Influence of Parturition upon the Creatinine Concentration of the Blood.

During pregnancy the concentration of creatinine in the maternal plasma (or blood) does not normally exceed 1.2 mg. per 100 cc.[5] This limit, it may now be noticed, is passed in one-third of all determinations made at the end of the second stage of labor. The maximum here attained (2.2 mg.) is practically double that of the antepartum period; and whereas the average of ten results secured during pregnancy was only 0.92,[5] that of the present series is 1.15. The six cases (Nos. 13 to 18) responsible for these differences were, as much as the others, clinically normal individuals. The high concentration of creatinine which they showed appears therefore to have been a physiological phenomenon. Its acceptance as such would imply that a temporary accumulation of creatinine in the blood is a normal, if perhaps not invariable, consequence of the act of parturition. Of course, before such a conclusion could be taken as safely established, it would be necessary to show, by repeated determinations upon individuals, that the creatinine concentration during labor is actually higher than that of the periods immediately before and after. Systematic observations capable of affording such a demonstration have not yet been undertaken, but the few pertinent data in our possession do indicate precisely the effect postulated. In Cases 9, 13, and 15 the creatinine of the plasma was determined not only at parturition but also on the 9th to 11th day of the puerperium. In each instance its concentration was found to have fallen; for the first from 1.00 to 0.87, for the second from 1.26 to 0.70, and

for the third from 1.51 to 0.91. The observation on Case 9 suggests that even those figures of the table which fall within the normal range may represent a rise above the subject's ordinary level.

The extent of the rise in individual cases does not, as far as our limited data enable us to judge, in any way depend upon the age of the subject, the duration and character of the anesthesia, or the length of the labor. The only variable with which it appears to possess some regularity of relation is the number of previous confinements. Those cases which showed an unusual concentration of creatinine were all (with the possible exception of Case 17) in their first labor. Not only so, but among all the subjects examined there are only two primiparas out of ten (possibly eleven) who show less than 1.0 mg. per 100 cc., while of the seven multiparas not one has more. The cases are too few in number to support a generalization upon the point; but their tabulation may be admitted at least to suggest that the influence of labor upon the blood creatinine is greatest among primiparas.

The increased concentration at parturition, which our data suggest as normal for creatinine, is apparently not shared by many of the other non-protein nitrogenous constituents of the blood; for Slemons and Morriss[6] have shown that the total non-protein nitrogen and specifically the urea remain within normal limits, while Morse[12] observed no change during labor in the concentration of the amino-acids. Uric acid, on the other hand, presents striking analogies of behavior with creatinine. For, although Slemons and Bogert[13] found its concentration in the majority of uncomplicated cases normal, they demonstrated in several instances a definite increase consequent upon labor, and observed the occasional occurrence in primiparas, although not in multiparas, of values abnormally high. It is of interest that the blood sugar also, according to Morriss,[14] although normal during pregnancy and the puerperium, rises at parturition just as we believe the creatinine to do. Three then of the blood constituents show, either regularly or occasionally, an increase of concentration

[12] Morse, A., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 199.

[13] Slemons, J. M., and Bogert, L. J., *J. Biol. Chem.*, 1917, xxxii, 63. See also Kingsbury, F. B., and Sedgwick, J. P., *ibid.*, 1917, xxxi, 261.

[14] Morriss, W. H., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 140.

during labor. It is perhaps worthy of note, that each of these three is believed to be connected, in one way or another, with muscular work. Morriss, accordingly, attributes the behavior of blood sugar during labor directly to the voluntary efforts of the mother during the second stage; and Slemons and Bogert incline to bring the concentration of uric acid also into relation with the duration and severity of the labor. Between this factor and the creatinine content of the blood we have been unable in our short series of cases to discover any apparent connection. If it exists, it has been obscured by other intercurrent effects. In the case therefore of creatinine the immediate cause of an increased concentration at parturition must remain a matter of speculation.

Placental Transmission of Creatine.

The unmodified Folin method for creatine, whether applied to whole blood or to plasma, is affected, as we have found,[2] by a large and, for the single determination, incalculable error. This may render doubtful the significance of data for creatine in individual cases, but does not seriously affect the value, as a basis for comparison, of the averages for a reasonably large series like the present.

The *plasma* averages, which alone are of immediate significance for our central problem, are in mother and fetus almost identical. Between individual results, whether these lie above or below the general average, there is noticeable a correspondence, which is often nearly, or even quite, as exact; while of a really conspicuous lack of agreement there are not more than one or two instances. As regards the creatine of plasma, therefore, our original report,[1] confirmed as it has been by the observations of Plass,[3, 4] requires no modification. We infer accordingly that the placental transmission of creatine, like that of creatinine, is a process of simple diffusion through structures equally permeable in either direction. It may be noted as not inconsistent with this view that, as far as there appears to exist any tendency at all to the establishment of a systematic difference of concentration, it operates again, as with creatinine, in favor of a slightly higher level in the fetal plasma.

Absolute Creatine Content of the Plasma at Parturition.

The conclusion reached is independent of the absolute accuracy of the creatine figures, the argument assuming only that, as is probable, the average error in maternal and fetal plasma is the same. The extent of that error we have estimated[2] as such that the true average for plasma is roughly one-fourth of the average found. The averages of the present series, corrected accordingly, become 0.52 and 0.54. If these values are added to those for creatinine, we find that the "total" creatinine of our cases averaged 1.67 for the mother, and 1.75 for the child. It must be rather more than a coincidence that Plass,[4] utilizing an improved method of analysis,[4,9] found the average "total" creatinine of twelve specimens of maternal and fetal plasma to be respectively 1.70 and 1.73. As the preformed creatinine of these particular specimens was not determined, we are left in ignorance of their content of actual creatine; but for an earlier series of nine cases Plass[3] has given figures indicating an average creatine of 0.76 for maternal and 0.86 for fetal plasma. These are estimates which are probably at least a little too high,[15] and which in any case are but slightly in excess of our own. Considering that the latter can be offered as approximations only, their substantial agreement with the independent observations quoted is extremely gratifying.

We have shown elsewhere[5] that the (corrected) plasma creatine of normal individuals with creatine-free urine does not exceed, on the average, 0.41 mg. per 100 cc.[16] Average values higher than that are yielded only by groups of individuals excreting creatine. The value 0.52, found for the mothers of the present series, is practically identical with that yielded by the twenty cases of creatinuria which we previously encountered among forty-six healthy subjects. We made no actual determinations of urinary creatine in the cases now reported, but, in view of the

[15] They were reached by the use of the original method of Folin. The experience of Wilson and Plass[9] with this method as applied to the determination of creatine in plasma has been less unfavorable than our own; for they found it to yield results not very much higher than those of their improved procedure.

[16] Actually it may, as Wilson and Plass[9] maintain, be a zero quantity; we are dealing here with approximations only.

regular occurrence of creatinuria during pregnancy,[17] it may be assumed to have been present in practically every one. The results upon parturient women, none of which were utilized in our previous paper, may therefore be taken as additional evidence for the tentative views therein expressed upon the relation of creatinuria to the concentration of creatine in the plasma.

Creatine of Whole Blood and of Corpuscles in Mother and Fetus.

The *whole blood* results for creatine, individually considered, are very irregular, but permit this generalization at least, that in a decided majority of instances the fetal blood contains a higher concentration of creatine than the maternal. There are a few cases of approximate equality, but only one (No. 5) where the fetal concentration is unequivocally the lower. The impression thus created takes definite form, with something at least of quantitative meaning, in the corrected averages.[18] According to these the maternal blood at parturition, with 3.45 mg. per 100 cc., shows practically the same concentration of creatine as that of a group of pregnant women, recently reported to contain 3.50 mg.[5] The blood of the fetus contains distinctly (about 23 per cent) more, reaching an average (4.25) higher than we have encountered in any other group of bloods. Since the plasma concentrations are practically identical, this might at first be supposed to indicate that the fetal corpuscles possess a greater accumulation of creatine than the maternal. This is not really the case. If from the average data for relative plasma volume, whole blood creatine and creatine of plasma, one calculates the creatine content of the corpuscles, it is found to be 8.2 mg. per 100 cc. for the mother, and 8.4 for the fetus. Apparently then the superior richness in creatine of the fetal blood is due entirely to the relative polycythemia which it almost invariably presents; and the approximate equality of fetal and maternal concentrations, already established for the plasma, is seen to extend also to the corpuscles.

The corpuscular creatine of various groups of normal men and

[17] Krause, R. A., and Cramer, W., *J. Physiol.*, 1910, xl, p. lxi. Krause, R. A., *Quart. J. Physiol.*, 1911, iv, 203.

[18] The corrected average for whole blood is reached by halving the average found.

non-pregnant women we have estimated at from 6.1 to 7.0 mg. per 100 cc.[5] The value now reached for parturient women is decidedly higher, and approximates rather that (9.1) found from 1 to 19 days antepartum in a smaller group of pregnant women. Our results suggest therefore that in the later stages of pregnancy there takes place a special accumulation of creatine in the red blood corpuscles. In this respect, as in so many others, they are in harmony with those of Plass.[4] The harmony extends even to certain quantitative details. We have calculated that in the ten cases reported by Plass the average "total" creatinine content of the corpuscles is 10.83 and 10.49 for mother and fetus respectively; the corresponding figures in our own cases (putting the true corpuscular as equal to the observed plasma creatinine) are 9.35 and 9.61. The differences are comparatively trivial, and the two estimates may be regarded as mutually confirmatory.

SUMMARY.

The concentrations of creatinine and creatine in the maternal plasma differ little, if at all, from those of the same substances in the fetal. In either case the average concentrations in these respective situations become, in a sufficiently large series, practically identical. If there is any perceptible tendency to a systematic difference, it is in the direction of a slightly higher concentration in the fetus. It is probable, therefore, that the placental transmission of creatinine and creatine is a simple process of diffusion.

Parturition is accompanied normally, although perhaps not invariably, by a temporary rise in blood creatinine. This effect appears to be illustrated most strikingly among primiparas.

The creatine content of the plasma at parturition corresponds with that found normally in association with creatinuria.

The concentration of creatine in the whole blood is generally greater in the fetus than in the mother; but the concentration in the corpuscles, as in the plasma, is practically the same in both.

The concentration of creatine in the corpuscles of parturient women is greater than in those of non-pregnant women or of men. It would appear that pregnancy is accompanied by a special accumulation of creatine in the corpuscles.

NUTRITIVE FACTORS IN ANIMAL TISSUES. II.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

(Received for publication, February 21, 1918.)

In the first paper[1] of this series we showed that beef skeletal muscle furnishes protein entirely adequate for the needs of growing albino rats. This tissue, however, was found to contain a far smaller proportion of the water-soluble vitamine than occurs in the liver tissue of the pig or such now familiar tested sources of this essential dietary constituent as yeast, corn germ, wheat embryo, or "protein-free milk." Reference was also made to Eddy's demonstration of the presence of the water-soluble vitamine in pancreatic tissue. When dried beef muscle formed he sole source of protein and water-soluble vitamine in the diet we observed that our animals did not grow for any considerable period, in fact they all soon declined unless some addendum of water-soluble vitamine from another source was supplied (see first paper, Chart I).

It was surprising, therefore, to discover the remarkably satisfactory growth of rats on a diet in which another closely related muscular tissue—pig's *heart*—furnished the sole source of protein, water-soluble, and in some cases fat-soluble, vitamine in the food.

The heart tissue was prepared by grinding fresh hearts from which the larger veins and arteries, fat, and connective tissue had been carefully removed. The finely comminuted cardiac muscle was then spread on pans, dried in a current of hot air, and ground to a fine powder.

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.

The food mixtures consisted of:

	A	B
	per cent	*per cent*
Pig's heart tissue, dried	19	19
Salt mixture[2]	4	4
Starch	54	54
Butter fat	0	9
Lard	23	14

The outcome of these feeding experiments is represented graphically in Chart I where it will be noted that those animals receiving butter fat in place of part of the lard grew the more vigorously.

Kidney tissue from the pig, likewise, has sufficed as the sole source of protein and vitamines, as is exemplified in Chart II, with animals receiving a diet of the following composition:

	per cent
Pig's kidneys, dried	22
Salt mixture[2]	4
Starch	51
Lard	23

The desiccated kidney tissue was prepared by finely comminuting the fresh kidneys (which had been freed from capsules and connective tissue), drying them in a current of hot air, and grinding to a fine powder.

The presence of the vitamines in this tissue has already been indicated elsewhere.[3]

With *brain tissue* from the pig excellent growth has been attained in the two rats for which the curves of growth are represented in Chart III. Here the brain served as the sole source of protein and water-soluble vitamine in food of the following composition:

	per cent
Pig's brains, dried	32.5
Salt mixture[2]	4.0
Starch	45.5
Butter fat	9.0
Lard	9.0

The brain was prepared by comminuting the fresh brains (previously freed from membranes and blood vessels), drying them in a current of hot air, and grinding the residue. Only the cerebrum was used.

[2] The salt mixture used is described in *J. Biol. Chem.*, 1917, xxxii, 317.

[3] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641; xxi, 179. McCollum, E. V., and Simmonds, N., *ibid.*, 1918, xxxiii, 55.

These dietary tests furnish excellent evidence of the "complete" quality of the proteins present in the brain as an organ.

The animals (cf. Chart V of the previous paper) showing the high efficiency of dried pig *liver* as the source of both protein and water-soluble vitamine for nutrition during growth have since rapidly reached adult size. In some cases this has occurred without the addition of any source of fat-soluble vitamine other than that contained in the tissue itself.

The presence of the fat-soluble vitamine in the liver tissue of the pig has been further confirmed by the use of the *liver oil* whereby the nutritive decline has been averted and the pathological manifestations which are the characteristic outcome of feeding a diet devoid of the fat-soluble vitamines have been cured.

This product was prepared by thoroughly extracting the finely ground dried liver with commercial ether about 15 times, using a fresh supply of ether each time. The ether was removed from the extract, and the residue, which was dark colored and tar-like in consistency, was dried over sulfuric acid in a vacuum desiccator.

The results of the administration of this liver oil are shown in Chart IV. At the point indicated by an arrow in the chart Rat 4503 began to manifest the eye disease which we first pointed out several years ago[4] and which has since been repeatedly described by others, both in the case of growing children and experimental animals. Complete relief followed within a few days after the administration of the liver oil. The outcome corresponds with the effect which we described some time ago as characteristic of the comparable use of cod liver oil.[5]

It is worthy of note that these results, which demonstrate the unlike value of different animal tissues as sources of the water-soluble vitamine, closely parallel the findings of Cooper[6] with respect to the effectiveness of different tissues in postponing the development of polyneuritis in pigeons fed on a diet of polished rice. He found that the liver, cardiac muscle, and cerebrum of the ox were much more efficient in preventing the onset of this disease than was the ox voluntary muscle.

[4] Osborne and Mendel, *J. Biol. Chem.*, 1913–14, xvi, 431.
[5] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 401.
[6] Cooper, E. A., *J. Hyg.*, 1912, xii, 436; 1914, xiv, 12.

The effect of varying the *proportions* of liver when used as the sole source of water-soluble vitamine in an otherwise adequate diet is strikingly shown in the growth curves exhibited in Chart V. The "basal" food mixture in these cases consisted of protein in the form of either casein or washed meat residue, a salt mixture, starch, butter fat, and lard, the supplementary desiccated tissues being used to replace part of the protein and starch. Whenever the diet contained 10 per cent of the liver tissue good growth always ensued. This was never the case when the proportion of liver tissue was reduced to 5 per cent; and rarely with 7 per cent. 10 per cent of desiccated cerebrum was inadequate.

Our experiments also demonstrate that the vitamines with which we were concerned are not destroyed by heat; for all of these preparations were subjected to a temperature of about 90° for several hours in the process of drying.

CHARTS.

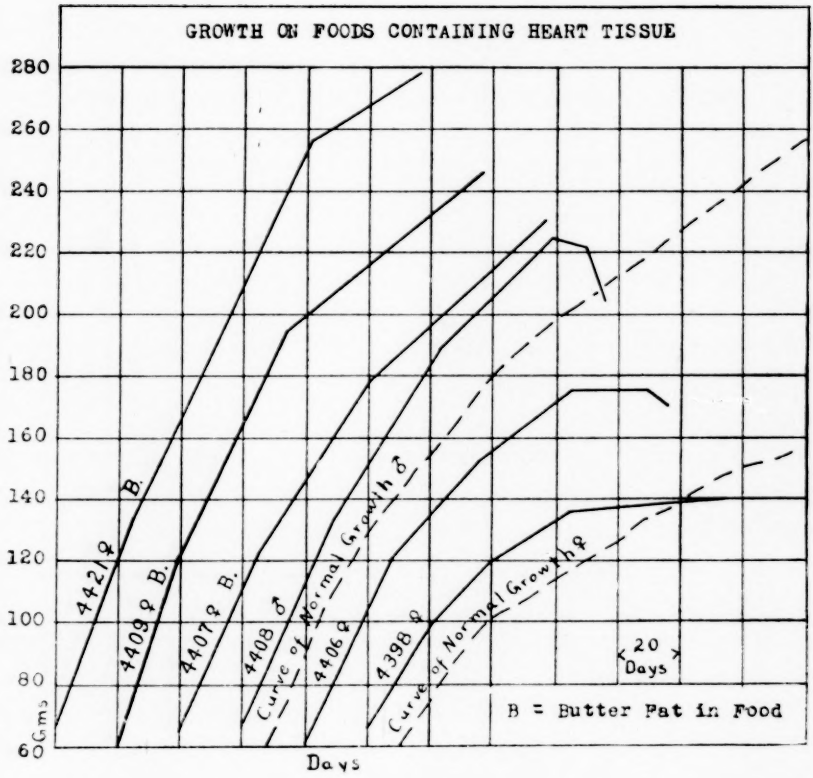

CHART I. Showing the growth of rats on diets containing desiccated *heart muscle* of the pig as the sole source of protein and water-soluble vitamine. The records of those animals which received additional fat-soluble vitamine in the form of butter fat are designated B. The composition of the foods is given in the text.

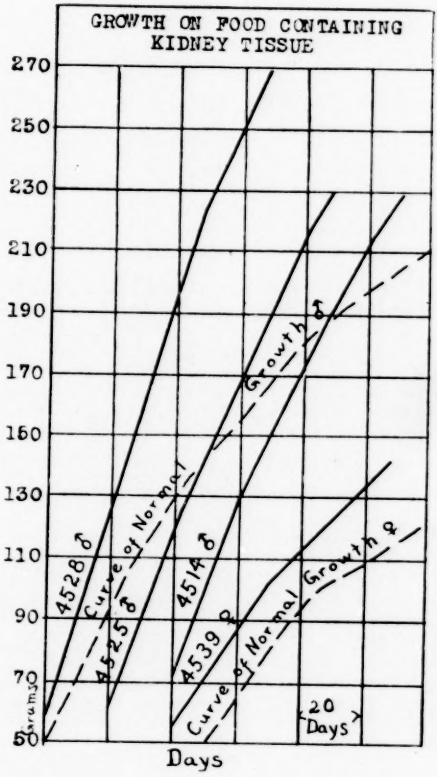

CHART II. Showing excellent growth of rats on a diet containing desiccated *kidney tissue* of the pig as the sole source of proteins and vitamines. The composition of the food is given in the text.

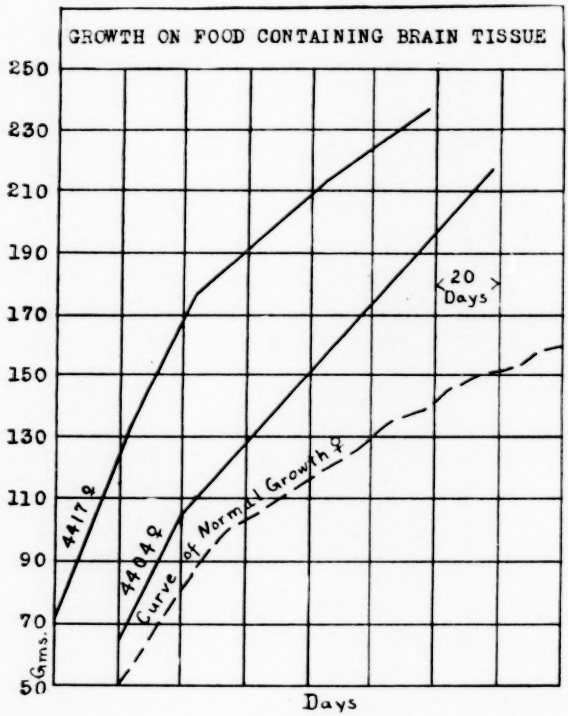

CHART III. Showing excellent growth of rats on a diet containing desiccated *brain tissue* of the pig as the sole source of protein and water-soluble vitamine. The composition of the food is given in the text.

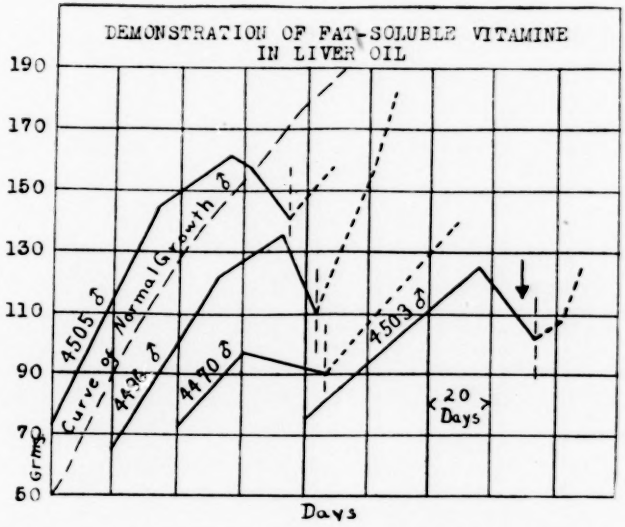

CHART IV. Showing the effect of *liver oil* in promoting the recovery of rats that had begun to decline in body weight on diets containing fat in the form of lard and accordingly deficient in the fat-soluble vitamine. The period of administration of liver oil is shown by a dotted line (- - - - - -). At the period indicated by the arrow, Rat 4503 manifested pronounced symptoms of eye disease which promptly disappeared after the use of the liver oil. The diet consisted of:

	Period 1 (solid line). *per cent*	Period 2 (dotted line). *per cent*
Casein	18	18
Protein-free milk	28	28
Starch	27	27
Liver oil		10
Lard	27	17

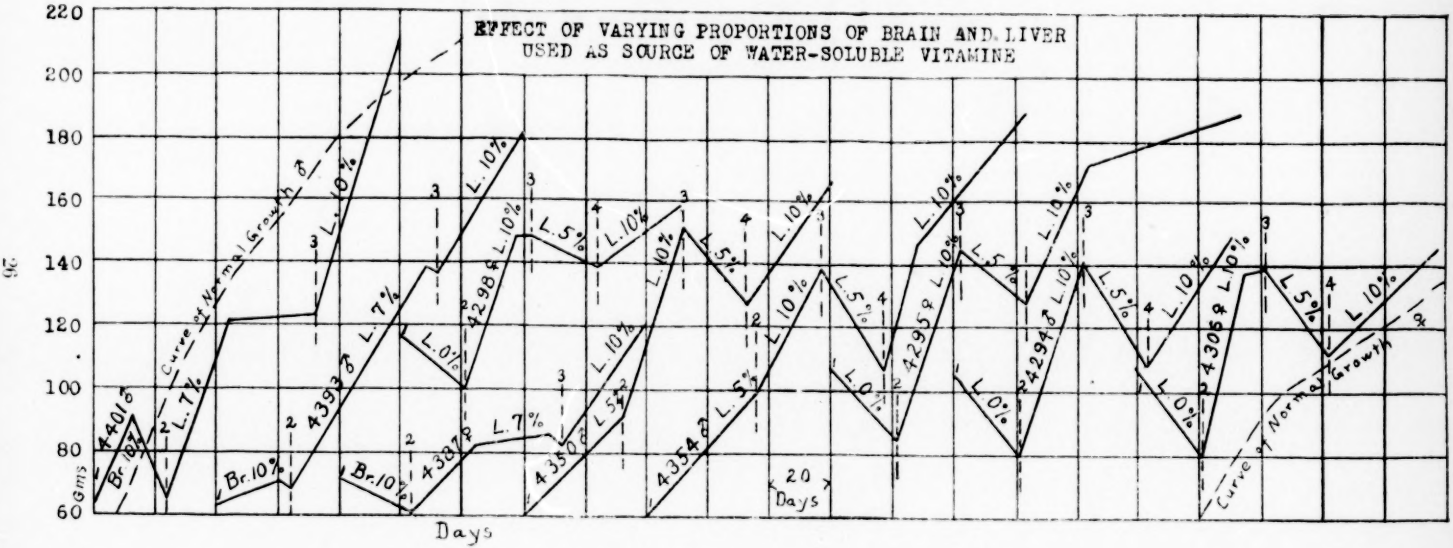
EFFECT OF VARYING PROPORTIONS OF BRAIN AND LIVER
USED AS SOURCE OF WATER-SOLUBLE VITAMINE
220
200
180
160
140
120
100
80
60
Gms
Days
20
Days
Curve of Normal Growth ♂
Curve of Normal Growth ♀
4401 ♂ Br 10% L 7%
4393 ♂ L 7%
4387 ♀ L 7%
4298 ♀ L 10%
4350 ♂ L 5%
4354 ♂ L 5%
4295 ♀ L 10%
4294 ♂ L 10%
4306 ♀ L 10%
Br 10%
L 10%
L 5%
L 0%

CHART V. Showing the comparative effect of the addition of *brain tissue* (Br) and of different proportions of desiccated *liver tissue* (L) as sources of the water-soluble vitamine supplementing an otherwise adequate food mixture. The contrast between 10 per cent and 5 per cent of the liver tissue is uniformly striking. The diets consisted of:

	Rats 4401, 4387, 4393.		
	Period 1.	Period 2.	Period 3.
	per cent	*per cent*	*per cent*
Casein	14	14	14
Dried pig's brain	10		
Dried pig's liver		7	10
Salt mixture*	4	4	4
Starch	50	50	47
Butter fat	18	18–9	9
Lard	4	7–16	16

	Rats 4298, 4294, 4295, 4306.			Rats 4350, 4354.	
	Period 1.	Periods 2 and 4.	Period 3.	Periods 1 and 3.	Periods 2 and 4.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Meat residue	18.5	11.5	15.0	18.5	11.5
Dried pig's liver		10.0	5.0	5.0	10.0
Salt mixture*	4.0	4.0	4.0	5.0	4.0
Starch	53.5	50.5	52.0	47.5	50.5
Butter fat	18.0	18.0–9.0	18.0	18.0	18.0–9.0
Lard	6.0	6.0–15.0	6.0	6.0	6.0–15.0

* The composition of the salt mixture used is given in *J. Biol. Chem.*, 1917, xxxii, 317.

THE TWO FORMS OF GLYCINE.

By K. GEORGE FALK and KANEMATSU SUGIURA.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Received for publication, February 21, 1918.)

INTRODUCTION.

Emil Fischer[1] found that glycine and alanine crystallized from water in plates which gave little or no acid chloride when treated with acetyl chloride and phosphorus pentachloride. Crystallized from water after the addition of alcohol, needles were formed which yielded over 50 per cent of the calculated quantity of acid chloride when treated similarly. No explanation was suggested for the difference in action or the isomerism of the two forms.

Method of Preparation.[2]

The needle form may be prepared by dissolving the glycine in a small quantity of water, heating, and then adding three to four times the volume of hot 95 per cent ethyl alcohol and cooling rapidly in an ice mixture. Methyl alcohol or acetone may be used in place of ethyl alcohol.

The plate form may be obtained by dissolving the glycine (needles or plates) in a small quantity of water and allowing the solution to evaporate in a vacuum desiccator over calcium chloride or sulfuric acid. In this way, well formed plates may be obtained.

If an insufficient amount of alcohol was added, or if crystallization took place slowly so that part of the alcohol evaporated, a mixture of plates and needles was obtained. These could readily be separated from each other by mechanical means. At times the plate form was obtained when the conditions apparently were

[1] Fischer, E., *Ber. chem. Ges.*, 1905, xxxviii, 2914.

[2] Thanks are due to Dr. James Senior of The Rockefeller Institute for Medical Research, who supplied the writers with a considerable quantity of partially purified glycine ester hydrochloride.

adjusted for the preparation of the needle form, and *vice versa;* but, as a rule, the crystal forms could be obtained as described. The hydrogen ion concentrations of the solutions between $10^{-3.0}$ and $10^{-11.0}$, adjusted with dilute hydrochloric acid and sodium hydroxide, exerted no influence on the crystal form. Several times the addition of ammonia to an aqueous solution resulted in the separation of needles where plates had been expected.

Decomposition Temperatures.

Glycine does not possess a sharp melting point, but decomposes as the temperature is raised. It was observed that the two forms began to decompose at different temperatures. These temperatures were determined in a Thiele melting point tube, containing sulfuric acid, with an ordinary 360° thermometer. The substances were dried in an electrically heated air oven for 3 hours at 100–103°, finely ground in an agate mortar, and placed in the customary capillary tubes. The temperatures given below are corrected. The determinations with the two forms were made simultaneously.

Color of substance.	Needles.	Plates.	Needles.	Plates.	Needles.	Plates.
	°C.	°C.	°C.	°C.	°C.	°C.
Light brown	230	212	222	212	223	211
Brown	232	216	232	227	233	227
Dark brown	235	222	235	231	235	233
Black	237	232	236	234	237	235
Began to vaporize	241	236	237	236	241	240

These results show that the needle form began to decompose at a higher temperature than the plate form.

Amino Nitrogen Determinations.

Van Slyke[3] had found that glycine treated with nitrous acid in the apparatus devised by him[3,4] gave a volume of gas corresponding to more than 100 per cent of nitrogen in the glycine. Under fixed conditions he obtained a fairly constant nitrogen con-

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185.

[4] Van Slyke, *J. Biol. Chem.*, 1912, xii, 275; 1913-14, xvi, 121.

tent of 19.9 per cent or 106.7 per cent of the calculated quantity. Longer treatment caused a greater evolution of gas, whose composition, however, was not given.

The micro-apparatus of Van Slyke was used in this work. The needle and plate forms were treated in the same way as far as possible. The experiments were carried out as follows: The substance was dried at 100–103° for 12 hours or in a vacuum desiccator over sulfuric acid. About 0.2 gm. was weighed out accurately, dissolved in a small amount of water, transferred to a 50 cc. volumetric flask, and diluted to the mark. A 2 cc. portion was used for each amino nitrogen determination. The same pipette was used for the solutions from both the needle and plate forms. The determination was made in the customary way with 6 minutes shaking of the deaminizing bulb. The rate of shaking was kept as constant as possible. The determinations were made as soon after solution was complete as possible, and at intervals thereafter. 25 cc. portions of the solutions were used for nitrogen determinations by the Kjeldahl method.

The results are shown in Table I. Column 2 shows the time after the glycine was dissolved which elapsed before the determination was made; Columns 3 to 5, the experimental values, Column 6, the weight of nitrogen corresponding to these, and Column 7, the percentage of nitrogen found. The results are given in groups, and at the beginning of each group are given the weight of glycine used in that series and the amount of nitrogen found by the Kjeldahl method.

These results show that the plate form of glycine when freshly dissolved gave a slightly larger amount of gas than the needle form, but that on standing in solution the value fell to that of the latter. The final value of both solutions was very nearly the same as that found by Van Slyke under the same conditions. The actual change in value for the plate form was small, it is true, but always in the same direction. From the number of experiments carried out (only a few of which are given in the table) and the strictly comparable experimental method of working, there appears to be no question of the occurrence of this change. At the same time, it must be stated that several times when the solutions were made up apparently with the plate form and a change in the value was expected, no such change occurred and

TABLE I.

Amino Nitrogen Determinations (Van Slyke) of the Two Forms of Glycine.

	Time after solution.	Temperature.	Barometric pressure.	Volume of gas.	Calculated as N.	N
	min.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*
Plate form.						
0.1881 gm. 18.7 per cent N.						
		19.0	766	0.12	0.069	
	45	19.7	766	2.87	1.65	21.0
	60	18.0	766	2.88	1.67	21.3
		19.1	766	0.12	0.068	
	310	19.0	766	2.76	1.59	20.2
	325	19.2	766	2.76	1.59	20.2
		16.2	764	0.12	0.071	
	1,440	19.2	764	2.72	1.56	19.8
	1,455	20.3	764	2.75	1.57	19.9
	1,470	20.5	764	2.76	1.57	19.9
		18.1	766	0.14	0.081	
	2,955	18.2	766	2.69	1.55	19.6
0.2014 gm. 18.4 per cent N.						
		16.2	764	0.11	0.062	
	10	16.0	764	2.94	1.71	20.4
	30	16.3	764	2.93	1.71	20.4
		16.5	760	0.12	0.069	
	1,158	16.5	760	2.91	1.68	20.0
	1,173	17.0	760	2.91	1.68	20.0
		20.1	766	0.16	0.092	
	4,218	19.1	766	2.92	1.68	19.7
	4,238	19.0	766	2.91	1.68	19.7
0.2016 gm. 18.5 per cent N.						
		17.9	768	0.12	0.070	
	40	18.2	768	2.98	1.73	20.6
	55	18.0	768	3.00	1.74	20.8
	70	18.0	768	2.98	1.73	20.6
		19.0	773	0.12	0.070	
	1,440	18.0	773	2.88	1.68	20.0
	1,455	19.0	773	2.89	1.68	20.0
		18.3	768	0.12	0.071	
	2,958	18.3	768	2.90	1.68	20.0
	2,973	18.1	768	2.90	1.68	20.0

TABLE I—*Concluded.*

	Time after solution.	Temperature.	Barometric pressure.	Volume of gas.	Calculated as N.	N
	min.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*
Needle form.						
0.2006 gm. 18.5 per cent N.						
		19.4	760	0.12	0.071	
	7	19.2	760	2.91	1.66	19.9
	27	19.2	760	2.91	1.66	19.9
		15.6	767	0.10	0.063	
	2,840	17.4	767	2.84	1.65	19.8
	2,858	18.0	767	2.86	1.66	19.9
0.2015 gm. 18.4 per cent N.						
		15.2	774	0.11	0.065	
	70	16.0	774	2.79	1.65	19.7
	85	17.0	774	2.83	1.66	19.8
	100	17.2	774	2.82	1.66	19.8
0.2083 gm. 18.5 per cent N.						
		15.2	756	0.10	0.060	
	7	15.3	756	2.94	1.70	19.7
	22	16.0	756	2.94	1.70	19.7
		17.9	769	0.12	0.071	
	2,940	17.7	769	2.93	1.70	19.6
	2,960	18.0	769	2.94	1.71	19.7

the same value as with the needle form was obtained. The needle form in solution did not change its amino nitrogen content, as determined, within reasonable time. In one experiment in which the solution stood for over a week, an increase in amino nitrogen was observed, but this was evidently due to gradual evaporation, the solution having been kept in a glass-stoppered bottle. The factors which govern the change of the plate form in solution until equilibrium between the different forms was attained were not studied further.

Action of Bromine.

The needle and plate forms of glycine were dried at 100–103°, ground uniformly, and exposed to bromine vapor in a desiccator

for 3 days, weighed rapidly, exposed for 3 days more, and weighed again. The following results were obtained.

	Needle form.	Plate form.
	gm.	*gm.*
Original weight	0.1001	0.1024
After 3 days	0.342	0.235
" 3 " more	0.354	0.249
Calculated weight $C_2H_5O_2N$ Br	0.2067	0.2115
" " $C_2H_5O_2N$ Br_2	0.3267	0.3342

Bromine determinations (as silver bromide) gave 58.5 per cent Br for the compound from the needle form and 49.0 per cent Br for the compound from the plate form; calculated for $C_2H_5O_2N$ Br, 51.6 per cent Br, for $C_2H_5O_2N$ Br_2, 68.1 per cent Br.

In this experiment therefore, each molecule of the plate form took up one atom of bromine and each molecule of the needle form took up two atoms of bromine. These compounds were red in color. On standing exposed to the atmosphere, the bromine was gradually given off again, leaving the colorless glycine.

Experiments with Alanine.

A number of experiments were made with alanine, but the results were not so interesting as with glycine. Two forms of alanine, plates and needles, may be prepared in the same way as the two forms of glycine were prepared. The plate form did not give as well defined crystal shapes as with glycine, but they were still markedly different from the needle form.

The relative decomposition temperatures of the two forms of alanine were similar to those of glycine, the plate form beginning to decompose at the lower temperature as shown by the following results.

	Needle form.	Plate form.
	°C.	°C.
Light brown	240	228
Brown	248	243
Dark brown		260
Began to vaporize	270	268

The amino nitrogen determinations for solutions of both the needle and plate forms of alanine did not change on standing. The results agreed with the value calculated for the simple formula. Possibly, the equilibrium between the two forms, if these differ chemically, is attained rapidly in solution.

When treated with bromine vapor both forms of alanine appeared to undergo decomposition.

DISCUSSION.

The results presented here make it evident that chemical differences, in addition to the difference noted by Fischer, exist between the two forms of glycine. The experimental evidence is not so sharp and clear cut as might be desired. At times when conditions were apparently suitable for one form, the other was obtained. The crystal forms even did not serve to distinguish between the two forms in some experiments. On the other hand, although the evidence is not altogether satisfactory, there appears to be no question that there is a difference between the two forms.

If an explanation of the difference is sought in structural formulas, $NH_2.CH_2.CO_2H$ and $NH_3.CH_2.CO.O$ may be suggested, although there is no direct chemical evidence available to distinguish between them.[5] With the corresponding trimethyl derivatives, betaine and the methyl ester of dimethylaminoacetic acid, the two isomers are distinct and well characterized.[6] If the similar structures apply to the two forms of glycine, then the differences in the two forms with hydrogen in place of methyl might be expected to be less pronounced and one form transformed into the other or equilibrium between the two established rapidly.

Recently Williams and Seidell[7] and Williams[8] alone found evidence to support the view that the presence of "vitamines" in foodstuffs was connected with the existence of isomeric or tautomeric forms of certain nitrogenous substances. Experiments

[5] Which of these formulas is to be assigned to glycine under definite conditions is still doubtful. Cf. Ley, H., and Hegge, H., *Ber. chem. Ges.*, 1915, xlviii, 84.

[6] Willstätter, R., *Ber. chem. Ges.*, 1902, xxxv, 584.

[7] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

[8] Williams, R. R., *J. Biol. Chem.*, 1917, xxix, 495.

with comparatively simple substances such as β-hydroxypyridine led them to the view that the betaine ring structure, N.C.C.O, was the active grouping in "vitamines." One of the structures for glycine also contains this grouping.

This investigation was suggested by some work which indicated that isomerism or tautomerism played an important part in protein reactions connected with enzymes.[9] The work was begun with glycine as a simple representative of the protein type.

The results obtained so far are of interest in connection with the possible existence of isomers in proteins and their derivatives and the different chemical, physical, and biological behaviors of these isomers.

SUMMARY.

The difference between the two forms of glycine, needle and plate, first noted by Emil Fischer in the formation of the acid chloride, was studied further.

The method of preparation, decomposition, temperatures, behavior with nitrous acid (Van Slyke method) and with bromine were studied and differences found to exist between the two forms.

Similar experiments with alanine showed less distinct differences between the two crystal forms of that compound.

[9] Falk, K. G., *J. Biol. Chem.*, 1917, xxxi, 97

A NEW METHOD FOR THE DIRECT NESSLERIZATION OF AMMONIA IN URINE.

By JAMES B. SUMNER.

(From the Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.)

(Received for publication, February 20, 1918.)

After the publication by Folin and Denis[1] of methods for the direct Nesslerization of ammonia in urine and in blood, the author attempted to obtain a supply of Merck's blood charcoal in order to use these new methods, and also in the hope of applying the principles involved to the determination of ammonia in muscle. There was no Merck's charcoal to be had. A substitute charcoal therefore was sought, and at the same time substances other than charcoal were investigated with regard to their ability to remove creatinine from urine, and creatine from muscle extracts. Thus far only one brand of charcoal with properties similar to those of Merck's has been found, an American made acid-washed bone charcoal sold by A. H. Thomas Company. This brand has been found to possess an unusual power of adsorption, and has been used with some success with urine, but is not satisfactory in some cases. It does not adsorb creatine, and cannot, therefore, be used for the determination of ammonia in muscle tissue.

Among a great many other reagents which have been tried, none has been found that removes creatine satisfactorily; but one, copper hydroxide, has been found to remove creatinine from urine rather well, and to carry down also all other substances which would interfere with the direct Nesslerization of ammonia.

The permutite method of Folin and Bell[2] has so simplified the determination of ammonia in urine that it seems hardly necessary

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

[2] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

to describe another method; but the use of copper hydroxide is rapid and easy and offers special advantages for time saving.

Copper hydroxide has long been used as a protein precipitant, and recently Van Slyke,[3] in determining acetone bodies in urine, has utilized it for the removal of glucose and other interfering substances. However, the author is not aware that it has ever been used expressly for the precipitation of creatinine.

For the direct Nesslerization of ammonia the urine is treated with a practically saturated solution of copper sulfate. Copper hydroxide is then precipitated by adding a sodium hydroxide solution of normality sufficient to bring the mixture rather close to the neutral point. At about the neutral point more than 90 per cent of the creatinine is precipitated, but when the reaction is either acid or alkaline the precipitation of both creatinine and copper is interfered with. The small amount of creatinine remaining in solution is not noticeable unless one is working with a sample of urine that contains less than 0.25 gm. of ammonia nitrogen per liter. In this case the Nesslerized solution is likely to have a slightly yellow tint, which will not interfere seriously with the reading, however, as repeated analyses have shown. Different samples of urine vary considerably in reaction, and it seemed at first desirable to use two different sodium hydroxide solutions, a stronger for acid urine and a weaker for alkaline urine; but it has been found that for all practical purposes one solution can be used, the reaction of the urine having no great effect upon the end-point. The normality of this solution should be 2.03. The copper sulfate solution should contain 298 gm. of pure crystallized copper sulfate per liter.

The sodium hydroxide solution should be free from iron and carbonate, and is best prepared from technical caustic soda according to the directions of Folin and Denis.

As copper sulfate sometimes contains ammonia, it should be tested for this by running a blank. If ammonia is present it can be removed by recrystallization.

The directions are as follows: Pipette into a large test-tube, preferably of 22 mm. diameter, exactly 10 cc. of the copper sulfate solution, 15 cc. of urine, and 10 cc. of the sodium hydroxide

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 455.

solution. Stopper immediately, and shake vigorously to mix. If the materials are not thoroughly mixed some of the copper may escape precipitation. Filter through an 11 cm. filter paper, covering the funnel with a watch-glass to prevent evaporation. Pipette into a 100 cc. volumetric flask the amount of filtrate that has been indicated advisable by a preliminary test (see below). Add one drop of a saturated solution of Rochelle salt to prevent the possible precipitation of the trace of copper that is present.[4] Dilute and Nesslerize with 10 cc. of the Nessler solution of Folin and Denis,[1] and compare in the colorimeter against a standard containing 1 mg. of ammonia nitrogen.

It has always appeared to the author that one of the chief sources of loss of time in determining ammonia by methods involving Nesslerization is the fact that one can seldom be certain of the amount of ammonia present in the unknown, and may therefore make the solution either too strong or too weak for a satisfactory comparison with the standard, necessitating another analysis. This can be avoided by Nesslerizing a minute portion of the filtrate from the copper hydroxide and roughly comparing the color obtained against a set of permanent standards. This portion is obtained by allowing the solution to drip directly from the funnel, where it is filtering, into a small test-tube. The filter funnels are made to deliver about 20 drops to the cubic centimeter by drawing the tips out in a flame until the outside diameter is about 2 mm., and cutting them off at this point. The funnels are then tested for the number of drops delivered per cc. Three drops of filtrate are taken for the test, diluted roughly to 6 cc., treated with about 1 cc. of Nessler's solution, and compared with the standards. The standards are made in the following manner: Dissolve about 12 gm. of crystalline ferric chloride and 5 gm. of crystalline cobalt nitrate in 200 cc. of water and filter. This solution will have a color that is approximately the same in quality as that of Nesslerized ammonia. Its intensity is de-

[4] The protective action of this reagent, and also of sodium citrate, is shown by the fact that, when one of these salts is present, ammonia diluted with the laboratory tap water yields on Nesslerization a colored solution that appears perfectly clear. There actually is some slight haziness to be observed, however, when a long column of the solution is compared with the ordinary standard against a black background.

termined by comparison in the colorimeter against 1 mg. of ammonia nitrogen freshly Nesslerized, and from the reading obtained one makes, by diluting, two solutions, one equivalent to 2 mg. of ammonia nitrogen (per 100 cc. of volume), and one equivalent to 1 mg. of ammonia nitrogen. The table below tells, in the first column, how much of these two solutions is taken for each of the six standards. The material is pipetted into small Jena test-tubes,[5] and made up to 7.5 cc. volume, after which the test-tubes are sealed with small round cover-glasses and sealing-wax. The second column in Table I tells how much Nesslerized ammonia nitrogen the standards are equivalent to, and the third column shows what volume of the unknown filtrate will yield about 1 mg. of ammonia nitrogen.

TABLE I.

Volume of iron-cobalt solution taken.	Nesslerized ammonia N represented.	Volume of filtrate containing 1 mg. of nitrogen.
cc.	*mg.*	*cc.*
7.5 (double strength).	0.15	1
7.5	0.075	2
5.0	0.05	3
3.0	0.03	5
1.5	0.015	10
1.0	0.01	15

Table II compiled from parallel ammonia determinations by aeration and by the new method indicates the accuracy of the results obtained.

[5] The alkali given off from ordinary glass causes the ferric chloride to deepen in color, after standing for a short time.

TABLE II.

Ammonia Nitrogen per Liter of Urine.

No.	Urine.	Copper method.	Aeration method.
		gm.	*gm.*
1	Normal	0.11	0.11
2	"	0.21	0.21
3	"	0.18	0.18
4	"	0.28	0.27
5	"	0.25	0.26
6	Diabetic	0.70	0.71
7	"	1.25	1.29
8	"	0.17	0.16
9	"	1.14	1.17
10	"	1.05	1.06
11	"	0.16	0.15
12	"	0.18	0.17
13	Albumin	0.93	0.92
14	"	1.30	1.30
15	"	0.22	0.20
16	"	0.32	0.38
17	"	0.22	0.24
18	Acetoacetic acid	0.61	0.60
19	" "	0.64	0.64
20	Alkaline fermentation	4.16	4.28

THE PRESENCE OF A GROWTH-PRODUCING SUBSTANCE IN CULTURES OF TYPHOID BACILLI.

By AUGUST J. P. PACINI and DOROTHY WRIGHT RUSSELL.

(From the Medical Laboratories of the American Telephone and Telegraph Company, New York, and the Bacteriological Laboratories of Pratt Institute, Brooklyn.)

(Received for publication, February 4, 1918.)

INTRODUCTION.

Soon after Funk announced the separation from rice polishings of supposed bases called by him "vitamines," and Suzuki, Shimamura, and Odake, working in the Orient, published corroborative evidence of the presence of a substance apparently similar to that described by Funk and called by the Japanese investigators "oryzanin," Edie, Evans, Moore, Simpson, and Webster in England reported the presence of similar bodies in brewer's yeast.

Without giving a survey of the development of the study of "vitamines," we may mention that such workers as Mendel, Osborne, Hopkins, Macallum, Seidell, Williams, and Eddy have confirmed the existence of food accessory substances which are capable of maintaining and increasing bodily weight and growth.

Clinical observers have long recognized that in certain infectious diseases growth is induced, apparently, by the infection. In typhoid fever, for example, the patient may grow from 1 to even several inches in height.

Yeasts and bacteria are so low in the zoological scale of life as to be virtually on the same plane; and there is no evident error of logic in inferring that the growth incident to bacillary infections may be due to substances, elaborated by the bacteria, such as the substances known to produce growth, which are elaborated by yeasts.

In the present paper we report experiments made to determine this point.

Technique.

Materials Employed.

1. Uschinsky's asparagine medium with agar.

Formula.

	gm.
Asparagine	3.4
Ammonium lactate	10.0
Sodium chloride	5.0
Magnesium sulfate	0.2
Calcium chloride	0.1
Acid sodium phosphate	1.0

The substances are dissolved in 1 liter of distilled water and 40 cc. of glycerol are added. To obtain a solid medium 1.5 per cent of agar is added. When prepared, the medium is submitted to fractional sterilization for 3 days. It is finally distributed in quart size Mason jars in such a manner as to form an evenly layered coat on the inner wall of the jar. Essentially these are large Esmarch rolls.

2. Anderson strain typhoid bacilli, obtained from the Department of Health, New York, and proved virulent by agglutinating them with known typhoid serum, diluted 1: 80.

3. Osborne and Mendel's casein diet, prepared from Merck's reagents, and consisting of:

	gm.
Casein	180
Starch	325
Granulated sugar	170
Agar	50
Lard	250
Salt mixture	25

The salt mixture has the following composition:

	gm.
$Ca_3(PO_4)_2$	10
K_2HPO_4	37
NaCl	20
Na citrate	15
Mg "	8
Fe "	2
Ca lactate	8

When this diet was prepared, the casein was extracted several times with boiling alcohol to insure the removal of any trace of vitamine that may have been present.

4. White rats, males, and as nearly the same weight as could be obtained from the animal dealer, were placed in separate cages holding wire meshed bottoms to provide for ready removal of feces. The food was placed in

empty ether tins with a small lateral aperture, the tin being hooked to the side, not resting upon the floor of the cage.[1]

5. Strains of Anderson type typhoid bacilli were planted in Uschinsky medium, liquid, and after incubation for 48 hours were poured into the jars prepared as described. By rolling the jar along the table top a few feet, the liquid plant was evenly distributed over most of the surface of the coating held by the jar. These plants were incubated for 72 hours when a relatively abundant growth of bacteria was obtained. Six jars were used. The cultures were scraped from the walls of the jars and transferred to Erlenmeyer flasks for further treatment. A volume of bacterial colonies corresponding to about 40 cc. was obtained from each jar. All the cultures were pooled together previous to extraction.

Extraction.—The collected bacteria were overlaid with about 300 cc. of absolute alcohol containing 0.8 per cent hydrochloric acid. They were allowed to stand 24 hours when the alcohol was filtered off and evaporated to dryness before the blast of an electric fan. The residue was taken up with about 100 cc. of distilled water, heated to 38°C., and filtered. The filtrate was again evaporated to dryness, taken up with a new volume of distilled water, filtered, and the filtrate made up to 300 cc. with water. This final filtrate, representing the fat- and protein-free extract of the typhoid bacteria was used in the experiments which follow, and will be referred to as "typhoid bacilli extract."

For purposes of control, 50 gm. of the solid Uschinsky medium were extracted in a similar manner, and the filtrate obtained is designated "medium extract."

Animal Experiments.

At the beginning of the tests, the weight of each rat was ascertained. Throughout the experiments the rats were weighed daily, and the food and water allotted per 24 hours measured and the amount recorded.

Four groups of tests were conducted, as follows:

Group 1.—Rats fed on the usual cabbage, carrot, and milk diet, to determine normal growth curves.

Group 2.—Rats fed on casein diet, to maintain them on a vitamine-free diet and at constant weight.

Group 3.—Rats fed on casein diet to which were added each day 5 cc. of medium extract, to insure that the medium, *per se*, did not induce growth.

Group 4.—Rats fed on casein diet to which were added daily 5 cc. of typhoid bacilli extract, to ascertain whether or not typhoid bacilli extracts contained vitamine.

There were three rats in each group.

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[1] This method was shown us by Dr. W. H. Eddy who used it in his experiments at the New York Hospital.

Results.

At the end of 30 days, the rats of Group 1, which were fed carrots, cabbage, and milk, had gained steadily in weight, increasing from 34, 45, and 49 gm., to 90, 107, and 97 gm., respectively. This is the usual rate of increase for young rats, and, therefore, represents a normal growth curve (Fig. 1).

Rats of Group 2 were fed on casein diet exclusively. Theoretically the casein diet should maintain the rats at constant weight. Experience shows, however, that the rats gradually lose weight

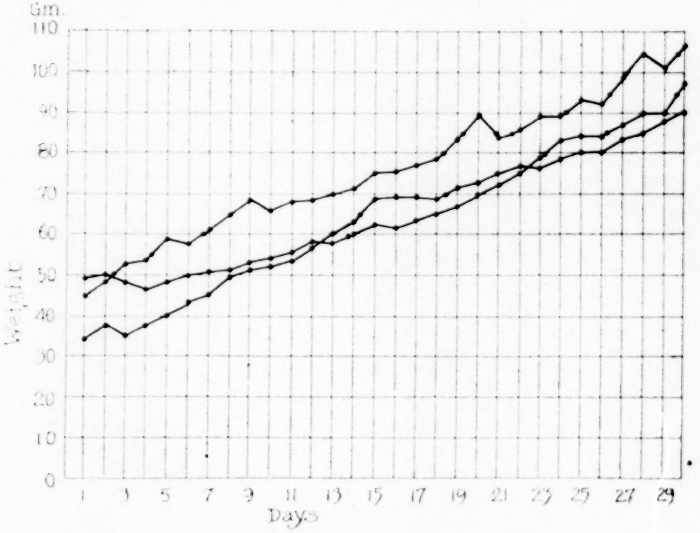

FIG. 1. Rats of Group 1, fed cabbage, carrots, milk, and distilled water.

at first, extending over a period of 2 weeks so gradually as to be of nearly constant weight, but after this time the loss is rapid and the animals die usually within 1 month. After allowing the animals to reach constant weight, that is after 2 weeks, test solutions of vitamine can be added to the basal ration and the effect noted.

Of the three rats fed exclusively on casein diet, one died after 18 days, the second after 24 days, the third after 27 days. The rats weighed respectively 48, 62, and 65 gm. at the start of the

tests. After 14 days, the weights were 40, 50, and 55 gm., respectively, and at death 12, 22, and 25 gm. (see Fig. 2).

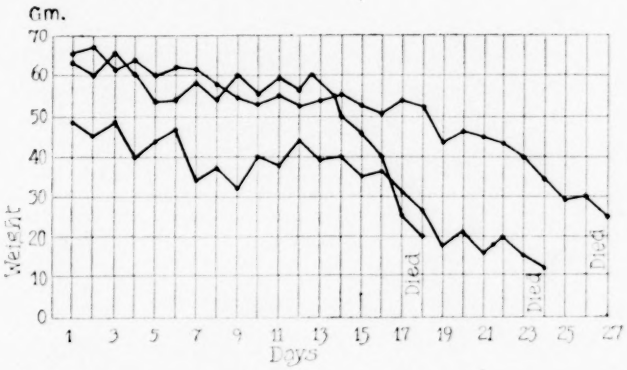

FIG. 2. Rats of Group 2, fed Osborne and Mendel's basal casein diet exclusively.

In the third group only two rats were fed the basal casein diet to which there were added each day 5 cc. of medium extract. Fig. 3 records the finding of how each rat continued to lose weight, indicating that the culture medium was void of vitamine substances.

Finally, the rats of Group 4 were fed casein diet to which were added daily 5 cc. of typhoid bacilli extract. The extract was not added until after the 14th day, however, as this time was allowed

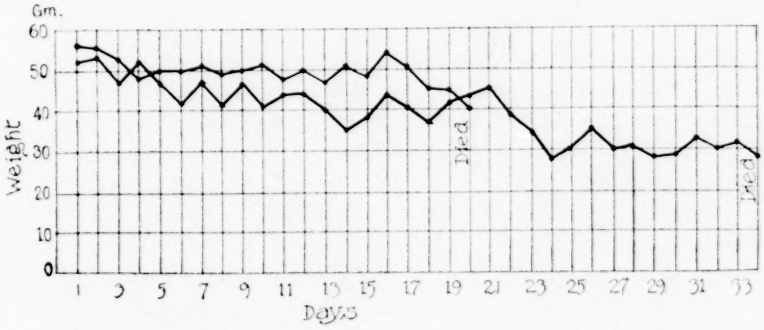

FIG. 3. Rats of Group 3, fed casein to which were added each day 5 cc. of culture medium extract.

to permit the animals to reach constant weight. It will be noticed that immediately upon adding the typhoid bacilli extract to the basal casein diet the rats gain in weight. Their gain is steady and simulates the curve obtained for normal growth. It will be further noticed that the conditions of the experiments are such as to preclude anything but the bacillary extract as responsible for the stimulation of growth.

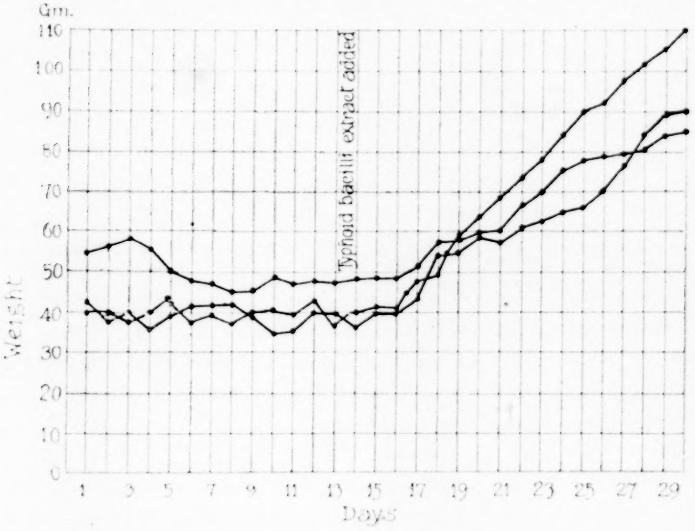

FIG. 4. Rats of Group 4, fed casein to which were added daily 5 cc. of typhoid bacilli extract.

Extracts containing vitamine have been shown to give a positive test, evidenced by the production of a blue color, with the Folin-Denis uric acid reagent. In this series, the medium extract reacted negatively, but the typhoid bacilli extract gave a markedly positive reaction.

Experiments are under way to ascertain whether other infectious organisms yield vitamines, and also, how to apply the vitamine formation therapeutically to the best interest of the infected patient. Already, evidence has been obtained that radical changes in the feeding of typhoid patients can be effected through this knowledge, and it is hoped that the conclusions reached will be of practical application.

CONCLUSION.

The typhoid bacillus, in growing, produces vitamine which can be isolated and identified by proper biological methods. The bearing on the effect of the same upon the general metabolism of typhoid patients, and indications of a scientific basis for new methods of feeding these cases, are under investigation.

STUDIES IN THE METABOLIC CHANGES INDUCED BY ADMINISTRATION OF GUANIDINE BASES.

II. THE INFLUENCE OF GUANIDINE UPON URINARY AMMONIA AND ACID EXCRETION.

By C. K. WATANABE.

(From the Laboratory of Pathological Chemistry, School of Medicine, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, February 1, 1918.)

In the altered metabolism conditioned by the absence of the parathyroids, Coronedi and Luzzatto (1) and Underhill and Saiki (2) were the first to find that the urine becomes alkaline and that the elimination of ammonia is simultaneously increased. MacCallum and Voegtlin (3) and Berkeley and Beebe (4) also reported an increased output of ammonia in the urine, relatively and absolutely to the total nitrogen, after parathyroidectomy in dogs. Cooke (5) likewise found a high ammonia output and alkaline urine. Later Wilson, Stearns, and Janney (6) showed that ammonia excretion and also the ratio of ammonia to total nitrogen was diminished in parathyroidectomized dogs. Upon the development of tetany, however, the elimination of ammonia and the ratio of ammonia to total nitrogen was increased. Burns (7) confirmed Wilson's work and stated that the increase in output of ammonia nitrogen corresponds to the onset of muscular tremors. The increased percentage of urinary ammonia was reported by Musser and Goodman (8) in postoperative tetany and by many other clinicians in idiopathic tetany.

The fact that the increased output of ammonia, above cited, was caused by the increased content of ammonia in the blood, was first reported by MacCallum and Voegtlin (3). Carlson and Jacobson (9) also demonstrated that the concentration of ammonia in the blood of animals in parathyroidectomy is greatly increased over that in the blood of normal animals. Jacobson (10) also showed that the concentration of ammonia in the blood of animals subjected to experimental ammonia tetany is practically equal to that of animals exhibiting symptoms of parathyroid tetany. Medwedew (11) also reported high ammonia in the blood of parathyroidectomized animals, whereas Greenwald (12) found no appreciable change in the blood ammonia under the same conditions.

The increased excretion of total nitrogen which occurs a few days after the appearance of tetanic symptoms was demonstrated by MacCallum and

Voegtlin (3). Most of the cases reported by Greenwald (12) and Cooke (5), Wilson, Stearns, and Thurlow (13), and Wilson, Stearns, and Janney (6) showed an increase after parathyroidectomy. Burns (7) reported that the excretion of total nitrogen rose slightly after the operation, but a few days later it fell to subnormal, which he attributes to the refusal of the animal to take all the food.

The increased excretion of ammonia in the urine and the simultaneous increase in the ratio of ammonia nitrogen to total nitrogen was reported by MacCallum and Voegtlin (3), Underhill and Saiki (2), Cooke (5), Berkeley and Beebe (4), Falta and Kahn (14), and Wilson, Stearns, and Janney (6). Burns (7), also reported the increased ratio after the development of tetany.

The fact that the reaction of urine becomes less acid and sometimes neutral or alkaline after parathyroidectomy was mentioned by Underhill and Saiki (2), Greenwald (12), Cooke (5), Burns (7), and Wilson, Stearns, and Janney (6).

From the literature it is certain that changes occur, especially in the nitrogen, and also in the acid excretion in the urine of animals subjected to parathyroidectomy. In the preceding paper of this series it was demonstrated that the increase of guanidine in the animal body by subcutaneous injection, induced symptoms almost identical with those of tetania parathyreopriva and at the same time hypoglycemia which also appears in tetania parathyreopriva. These facts lead us to believe that the guanidine which increases in the animal body as a result of the lack of parathyroids may influence the metabolism of nitrogen and also the acid-base equilibrium. This work was intended to test the above view. Burns (7) stated that the symptoms produced in animals as a result of injecting guanidine hydrochloride resemble those of tetania parathyreopriva in the increased excretion of nitrogen. He also states that in some cases the ammonia is increased over the normal though there is no increase in the ratio of ammonia to total nitrogen. The urine, likewise, showed a tendency to become alkaline after the administration of the drug. His evidence, however, is not clear and his variations after parathyroidectomy are too small. Moreover, such a small dose of guanidine was used that most of the animals did not manifest the symptoms of tetania parathyreopriva. More definite information on the results of injection of guanidine hydrochloride are therefore desirable.

Methods.

Rabbits were employed in this investigation. They were kept in metabolism cages which were cleaned every morning. The 24 hour specimen of urine was collected each morning at 9 o'clock by squeezing the bladder of the rabbits. Toluene was used for a preservative. The 24 hour specimen of urine was made up to 250 cc. and the diluted urine used for determinations. Total nitrogen was estimated by the Kjeldahl method. Ammonia was determined by aeration with Folin's method. For the determination of acid excretion the method of Henderson and Adler (15) was used and Henderson's method (16) for hydrogen ion concentration. Results in which the pH is less than 4.9 are merely approximations. For the above two methods the precautions mentioned by Wilson, Stearns, and Janney (6) were observed. Duplicate determinations were made in most cases. The character of the food may influence the ammonia and the acidity and also the hydrogen ion concentration. Hence uniform conditions must be preserved.

Rabbits were kept in the cage 3 days previous to the injection and oats and water given freely. On the morning of the 4th day at 9 a.m. a sublethal dose (0.2 gm. per kilo of body weight) of guanidine hydrochloride was administered subcutaneously to three rabbits and these were observed for 6 days after injection. During this time oats and water were kept in the cage. In the following tables is shown the striking difference in the total nitrogen, ammonia nitrogen, acid excretion, and hydrogen ion concentration before and after injection of the drug.

There is a marked decrease of total nitrogen in the urine just 24 hours after injection and this decreased elimination is independent of the volume of urine, since the volume of urine does not markedly decrease but rather increases in some cases throughout the investigation. However, on succeeding days the elimination of nitrogen is increased over that of the normal period. The elimination of ammonia nitrogen is markedly increased during the 1st day after injection, much more increased during the 2nd day, but becomes normal in a few days. There is a relative and absolute increase of ammonia nitrogen, so that the ratio of ammonia nitrogen to total nitrogen is remarkably high as compared

TABLE I.

Rabbit 3.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.					
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*	
Oct. 27.....	1,700		77	0.623	0.014	2.2	0.8	6.2
" 28.....			92	0.812	0.018	2.2	1.0	6.2
" 29.....			81	0.763	0.010	1.3	1.7	5.3
" 30.....	1,740	0.2 at 9 a.m.	72	0.336	0.032	9.5	0.3	5.5
" 31.....	1,520		72	0.616	0.052	8.5	0	6.9
Nov. 1.....	1,560		128	1.274	0.031	2.4	0.5	6.0
" 2.....	1,560		76	0.910	0.027	3.0	0.6	6.0
" 3.....	1,560		81	0.987	0.013	1.3	1.1	5.3
" 4.....	1,540		68	0.840	0.013	1.6	1.1	5.9

TABLE II.

Rabbit 4.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.					
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*	
Oct. 27.....	1,860		40	0.630	0.015	2.4	1.1	5.7
" 28.....			49	0.700	0.042	6.0	0.7	6.3
" 29.....			48	0.735	0.027	2.5	1.0	6.2
" 30.....	1,800	0.2 at 9 a.m.	70	0.335	0.034	10.0	0.1	6.8
" 31.....	1,580		85	1.120	0.073	6.5	0	7.0
Nov. 1.....	1,580		100	1.211	0.053	4.4	0	6.8
" 2.....	1,540		111	1.449	0.063	4.3	0.1	7.0
" 3.....	1,520		91	1.645	0.028	1.7	0.6	6.4
" 4.....	1,480		57	1.190	0.022	1.8	0.9	6.2

TABLE III.

Rabbit 5.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.					
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	NH_3 N / Total N	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm*	*per cent*	*cc.*	
Oct. 27.....	2,920		111	0.847	0.008	0.9	1.7	5.0
" 28.....			158	1.267	0.017	1.3	2.1	4.8
" 29.....			*	1.367	0.022	1.6	2.0	5.7
" 30.....	3,200	0.2 at 9 a.m.	72	0.406	0.039	9.8	0	6.9
" 31.....	2,920		109	0.896	0.053	5.9	0.1	7.0
Nov. 1.....	2,820		133	1.225	0.054	4.4	0.2	6.6
" 2.....	2,740		135	1.400	0.020	1.4	0.9	6.1
" 3.....	2,720		117	1.360	0.015	1.1	0.8	6.1
" 4.....	2,680		97	1.155	0.017	1.5	0.9	5.4

* Figures lost.

with the normal condition in each rabbit. Another striking feature is the decreased titratable acidity, the urine in some cases becoming neutral. The hydrogen ion concentration decreases and on the 2nd day some of the urines appear neutral. The increased excretion of ammonia nitrogen was accompanied by the lowering of acid excretion and a diminution of hydrogen ion concentration.

It was observed that, for a day or two after the injection of the drug, the rabbits do not eat, which results in a fasting condition that may have some effect on metabolism (17, 18). To take this condition into account the normal period of the rabbit was divided so that for 3 days the animal received oats and water, for 3 days water only, was fed well until the normal condition was obtained, then for 3 days was given oats and water, and on the 4th the guanidine was injected. The results of the experiment during the first 3 days after injection then were compared to the 3 days fasting normal period, and thus the effect on the animal due solely to the drug was obtained.

TABLE IV.

Rabbit 7.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine. Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH	Food.
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Nov. 6..	2,440		177	1.505	0.042	2.8	0.9	6.4	Oats and water.
" 7..			136	1.225	0.030	2.4	0.9	6.0	Same.
" 8..	2,420		84	1.008	0.035	3.5	1.1	6.3	"
" 9..	2,400		78	0.980	0.021	2.1	1.1	5.5	Water.
" 10..	2,320		142	1.456	0.017	1.2	1.7	4.7	"
" 11..	2,260		148	1.365	0.022	1.6	1.4	5.2	"
" 12..	2,200		202	1.225	0.020	1.6	1.4	5.3	"
									Fed well.
Nov. 30..	2,260		80	0.490	0.010	2.0	0.4	4.7	Oats and water.
Dec. 1..			75	0.651	0.008	1.2	0.7	4.7	Same.
" 2..	2,200		102	0.882	0.014	1.6	0.9	4.5	"
" 3..	2,185	0.08 at 9 a.m.	53	0.784	0.014	1.8	1.0	4.4	Water.
" 4..	2,020		197	0.994	0.015	1.5	0.8	4.4	"
" 5..		0.12 at 9 a.m.	23	0.077	0.015	19.5	0	7.0	"

As shown in Tables IV to X—first period of estimation—the excretion of total nitrogen increases after fasting owing to the increased catabolism of body proteins. The excretion of acid is also increased. However, there is almost no change in ammonia nitrogen. Distinct differences appear after the administration of the drug. The ammonia nitrogen is greatly increased and at the same time the ratio of ammonia to total nitrogen is very much increased. This means that the increase in the excretion of total nitrogen does not keep pace with the increase in ammonia nitrogen. As mentioned before, the total nitrogen is decreased in 24 hours after injection. The acid excretion and hydrogen ion concentra-

TABLE V.

Rabbit 8.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.						Food.
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine 0.1 N solution.	pH	
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Nov. 6..	1,280		48	0.490	0.010	2.0	0.5	5.2	Oats and water.
" 7..			62	0.602					Same.
" 8..	1,240		40	0.511	0.013	2.5	0.6	4.7	"
" 9..	1,180		57	0.630	0.006	1.0	0.8	4.7	Water.
" 10..	1,120		80	0.896	0.008	0.9	1.2	4.4	"
" 11..	1,080		75	0.840	0.010	1.2	1.2	4.4	"
" 12..	1,040		74	0.712	0.010	1.4	1.1	4.4	"
									Fed well.
Nov. 30 .	1,280		45	0.385	0.007	1.8	0.7	4.7	Oats and water.
Dec. 1..			45	0.420	0.011	2.6	0.7	4.5	Same.
" 2..	1,280		62	0.539	0.011	2.0	0.8	5.3	"
" 3..	1,200	0.15 at 9 a.m.	34	0.350	0.037	9.7	0	7.0	Water.
" 4..	1,140		54	0.630	0.035	5.6	0.1	7.0	"
" 5..	1,120		58	0.770	0.022	2.9	0.9	4.9	"
" 6..	1,100		64	0.602	0.021	3.5	0.9	5.6	"

tion were also decreased distinctly as usual where the sublethal dose was administered. The subtoxic dose (small dose) was tried on Nos. 7, 10, and 17. In this case the change in the urine does not differ from the fasting condition in 24 hours after injection. Whereas the other animals in which large doses of the drug were injected showed some degree of tetany, the above rabbits exhibited no symptoms of tetany. Nos. 7 and 10 were again injected with larger doses and this time the usual results were manifested. From the 2nd day after the administration of the drug the sudden increase of total nitrogen excretion was noted, but the percentage increase is no larger than during the normal

TABLE VI.

Rabbit 9.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine. Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	NH_3 N / Total N	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH	Food.
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Nov. 23..	2,880		136	0.805	0.014	1.7	1.1	6.0	Oats and water.
" 24..			115	1.120	0.013	1.2	1.7	5.5	Same.
" 25..	2,740		121	1.274	0.008	0.6	1.8	4.9	"
" 26..	2,720		151	1.624	0.008	0.5	2.1	4.8	Water.
" 27..	2,640		215	1.855	0.013	0.7	2.2	4.9	"
" 28..	2,620		202	1.660	0.014	0.8	1.9	4.8	"
									Fed well.
Dec. 8..	2,840		82	0.847	0.011	1.3	2.5	4.7	Oats and water.
" 9..			82	0.784	0.011	1.4	1.8	4.6	Same.
" 10..	2,720		115	0.861	0.012	1.4	2.0	4.6	"
" 11..	2,500	0.2 at 9 a.m.	117	0.350	0.028	8.0	0.2	5.7	Water.
" 12..	2,380		132	0.840	0.048	5.7	0.5	5.8	"
" 13..	2,320		107	0.875	0.035	4.0	0.6	5.6	"
" 14..	2,220		140	1.715	0.024	1.4	1.2	5.5	"

fasting period in most cases. The large dose of injected drug either induces some change in protein metabolism or the kidney may be less permeable to the nitrogen in such a condition.

TABLE VII.

Rabbit 10.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.						Food.
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH	
1917	gm.	gm.	cc.	gm.	gm.	per cent	cc.		
Nov. 23	2,020		45	0.490	0.008	1.6	0.7	5.9	Oats and water.
" 24			60	0.780	0.011	1.4	1.2	5.4	Same.
" 25	1,940		51	0.868	0.012	1.4	1.2	6.4	"
" 26	1,920		54	0.903	0.008	0.9	1.4	5.0	Water.
" 27	1,840		67	1.155	0.022	1.9	1.9	6.0	"
" 28	1,800		79	1.407	0.013	0.9	1.3	5.5	"
									Fed well.
Dec. 8	2,010		32	0.392	0.013	3.3	1.1	4.8	Oats and water.
" 9			44	0.630	0.014	2.2	1.3	5.0	Same.
" 10	1,940		48	0.686	0.017	2.5	0.9	5.7	"
" 11	1,660	0.06 at 9 a.m.	66	0.784	0.019	2.4	0.8	5.5	Water.
" 12	1,620	0.15 at 9 a.m.	59	0.413	0.088	21.2	−0.4	7.3	"
" 13	1,620		106	0.763	0.091	11.8	−0.3	7.0	"
" 14	1,560		65	0.728	0.041	5.6	0.1	6.9	"
" 15	1,540		73	1.050	0.054	5.1	0.1	7.0	"

TABLE VIII.

Rabbit 17.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.						Food.
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	NH_3 N / Total N	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH	
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Dec. 23..	2,160		25	0.448	0.020	4.5	−0.1	7.2	Oats and water.
" 24..			18	0.210	0.034	16.3	0	7.0	Same.
" 25..	2,120		57	0.567	0.035	6.2	0.8	5.7	"
" 26..	2,080		60	0.637	0.017	2.7	1.3	4.8	Water.
" 27..	2,010		36	1.015	0.015	1.5	1.6	4.8	"
" 28..	2,000		58	0.966	0.015	1.6	1.6	4.7	"
									Fed well.
1918									
Jan. 8..	2,205		30	0.721	0.011	1.5	1.6	5.0	Oats and water.
" 9..			33	0.850	0.008	0.9	2.0	4.9	Same.
" 10..	2,210		32	0.770	0.007	0.9	1.6	4.9	"
" 11..	2,140	0.1 at 9 a.m.	39	0.805	0.010	1.2	1.4	4.7	Water.
" 12..	2,000		79	0.805	0.028	3.5	0.8	5.7	"
" 13..	1,940		58	0.882	0.014	1.6	0.7	4.5	"

TABLE IX.

Rabbit 18.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine.						Food.
			Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc. of diluted urine. 0.1 N solution.	pH	
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Dec. 23..	2,360		58	0.672	0.011	1.6	1.2	4.5	Oats and water.
" 24..			73	1.015	0.014	1.4	1.5	4.4	Same.
" 25..	2,260		68	0.994	0.011	1.1	2.1	4.4	"
" 26..	2,180		78	1.204	0.013	1.1	1.7	4.4	Water.
" 27	2,140		78	1.323	0.008	0.6	1.8	4.4	"
" 28..	2,080		74	0.861	0.011	1.3	1.8	4.4	"
									Fed well.
1918									
Jan. 8..	2,280		60	0.661	0.013	2.0	1.4	4.8	Oats and water.
" 9..			65	0.850	0.011	1.3	1.8	4.8	Same.
" 10..	2,180		75	0.805	0.011	1.4	1.6	4.7	"
" 11..	2,000	0.2 at 9 a.m.	115	0.406	0.032	7.9	0.5	4.9	Water.
" 12..	1,860		84	1.085	0.040	3.7	0.2	6.0	"
" 13..	1,820		81	0.924	0.014	1.5	0.3	4.5	"

TABLE X.

Rabbit 19.

Date.	Body weight.	Guanidine hydrochloride per kilo.	Urine. Volume in 24 hrs.	Total nitrogen.	Ammonia nitrogen.	$\frac{NH_3\ N}{Total\ N}$	Titratable acidity in 10 cc of diluted urine. 0.1 N solution.	pH	Food.
1917	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*cc.*		
Dec. 23..	1,880		116	0.784	0.010	1.3	0.2	5.8	Oats and water.
" 24..			75	0.735	0.015	2.0	0.7	4.7	Same.
" 25..	1,720		53	0.770	0.013	1.7	0.8	4.6	"
" 26..	1,680		68	0.770	0.011	1.4	1.0	4.6	Water.
" 27..	1,590		77	0.917	0.011	1.2	1.4	4.7	"
" 28..	1,560		71	0.875	0.010	1.1	1.4	4.7	"
									Fed well.
1918									
Jan. 8..	1,660		43	0.661	0.014	2.1	0.9	4.7	Oats and water.
" 9..			32	0.630	0.014	2.2	0.8	5.7	Same.
" 10..	1,609		31	0.670	0.013	1.9	1.0	4.7	"
" 11..	1,460	0.15 at 9 a.m.	55	0.532	0.022	4.1	0.5	5.8	Water.
" 12..	1,380		82	0.826	0.017	2.1	0.6	5.7	"
" 13..	1,305		88	1.344	0.008	0.6	1.4	5.5	"

SUMMARY.

1. The introduction of sublethal doses of guanidine hydrochloride induces sudden diminution of total nitrogen in the urine in 24 hours, but after successive days it increases again, as in most cases in the fasting period in the same animal.

2. The ammonia elimination increases markedly after the administration of large doses of the drug. The ratio of ammonia to total nitrogen is increased greatly.

3. The acid excretion is markedly lowered in some cases the urine becoming neutral or alkaline after injection of the drug.

4. A decrease in hydrogen ion concentration occurs after the injection of the drug.

BIBLIOGRAPHY.

1. Coronedi, G., and Luzzatto, R., *Arch. ital. biol.*, 1907, xlvii, 286.
2. Underhill, F. P., and Saiki, T., *J. Biol. Chem.*, 1908–09, v, 225.
3. MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.
4. Berkeley, W. N., and Beebe, S. P., *J. Med. Research*, 1909, xx, 149.
5. Cooke, J. V., *J. Exp. Med.*, 1911, xiii, 439.
6. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *J. Biol. Chem.*, 1915, xxiii, 123.
7. Burns, D., *Quart. J. Exp. Physiol.*, 1916, x, 361.
8. Musser, J. H., and Goodman, E. H., *Univ. Penn. Med. Bull.*, 1909, xxii, 85.
9. Carlson, A. J., and Jacobson, C., *Am. J. Physiol.*, 1909–10, xxv, 403.
10. Jacobson, C., *Am. J. Physiol.*, 1910, xxvi, 407.
11. Medwedew, A., *Z. physiol. Chem.*, 1911, lxxii, 410.
12. Greenwald, I., *Am. J. Physiol.*, 1911, xxviii, 103.
13. Wilson, D. W., Stearns, T., and Thurlow, M. DeG., *J. Biol. Chem.*, 1915, xxiii, 89.
14. Falta, W., and Kahn, F., *Z. klin. Med.*, 1912, lxxiv, 108.
15. Henderson, L. J., and Adler, H. M., *J. Biol. Chem.*, 1909, vi, p. xxxviii.
16. Henderson, L. J., *J. Biol. Chem.*, 1911, ix, 403. Henderson, L. J., and Palmer, W. W., *ibid.*, 1912–13, xiii, 393.
17. Osterberg, E., and Wolf, C. G. L., *Biochem. Z.*, 1907, v, 304.
18. Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 165.

STUDIES IN THE METABOLIC CHANGES INDUCED BY ADMINISTRATION OF GUANIDINE BASES.

III. THE RELATION BETWEEN THE TETANOID SYMPTOMS OF GUANIDINE ADMINISTRATION AND THE CONDITION OF ACIDOSIS.

By C. K. WATANABE.

(From the Laboratory of Pathological Chemistry, School of Medicine, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

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It is a fact that in parathyroidectomized animals the excretion of ammonia is increased at the same time that ammonia appears in large quantity in the blood. As evidence for this statement two possibilities have been considered. First, as a result of defective oxidation in the body acid bodies are formed. Ammonia is formed from protein to neutralize these acids. Second, accelerated protein metabolism produces an excess of ammonia or the depressed liver function results in an inability to transform ammonia formed in protein metabolism into urea. Carlson and Jacobson (1) demonstrated the marked depression of the ammonia-destroying power of the liver in completely parathyroidectomized animals as compared with normal animals in perfusion experiments. They thought that the increase of ammonia in this condition is therefore due, at least in part, to liver depression rather than to acidosis independent of the liver. Wilson, Stearns, and Janney (2) state in their experiment that the elimination of ammonia and the ratio of ammonia to total nitrogen increased and that the acid and hydrogen ion concentration increased after the attack of acute tetany. They believe that after acute and chloric tetany an acidosis may occur. Wilson, Stearns, and Thurlow (3) put forward the idea that after parathyroidectomy a condition of alkalosis may develop which is neutralized by acid products formed by muscular activity incident to the tetany. An acidosis condition may result after the period of acute tetany. Their experiments were performed with the dissociation constant of oxyhemoglobin and hydrogen ion concentration of the blood and alveolar carbon dioxide tension. Underhill and Saiki (4), Cooke (5), and Wilson, Stearns, and Janney (2) found abnormal acid, such as diacetic acid, in the

urine in cases of tetania parathyreopriva with the development of tetany. In postoperative tetany and idiopathic tetany the abnormal acid was found.

After parathyroidectomy the acid reaction of the urine diminishes and the reaction becomes neutral or alkaline with a decrease in hydrogen ion concentration. This fact shows that the excretion of phosphate, at least acid phosphate, may be decreased in the urine after parathyroidectomy. Greenwald (6) found a marked retention of phosphates appearing on the first few days after parathyroidectomy. In all of six experiments the phosphate excretion falls to less than 50 per cent of its former amount and this condition is not accompanied by an increase in the elimination of phosphate in the feces. In normal condition we would expect the excretion of phosphate to increase in the excess of muscular activity (7, 8, 9) as in tetany. Greenwald (10, 11) reported that the retention of phosphate, which is insoluble in the usual lipoid solvents but soluble in a mixture of dilute acetic or hydrochloric or picric acids, was a fact. The increase of inorganic phosphorus (12) in the blood together with the decreased acidity of the urine in which monobasic phosphate is the main substance which is in the excretion of acid, may give us a picture of the acidosis in the body since the change from dibasic to monobasic phosphate is the normal mechanism by which the body gets rid of acid (13).

The above events show that in tetania parathyreopriva, acidosis may exist, and since, after injection of guanidine hydrochloride, almost the same evidences appear in the urine (14), guanidine may cause acidosis in the body.

Methods.

For the detection of the condition of acidosis several laboratory methods are available. The hydrogen ion concentration of the whole blood was obtained by Marriott's method (15). At the same time the CO_2-combining power of the plasma by Van Slyke's method (16) with the large size (17) apparatus was determined. Rabbits also were employed and about 7 cc. of blood were drawn from the jugular vein with the precaution mentioned by Van Slyke. The figures in the tables represent the number of cc. of CO_2 bound as bicarbonate in 100 cc. of plasma, reduced to 0° and 760 mm. Hg. The rabbits were fed freely with oats and water before and during the experiment.

The rabbits usually do not take food or water for 24 hours after the injection of guanidine. This may influence the reaction of the blood, and the spontaneous nephritis (18, 19, 20) in some cases is another obstacle. The above two factors make the results unreliable. To overcome these obstacles the following procedure

was adopted. Five normal rabbits were examined for the comparison with the rabbits which were injected with guanidine. In each rabbit the first blood sample was drawn at 2 p.m. and then the rabbit was deprived of food and water until the afternoon of

TABLE I.

Variation of CO_2 Capacity and of pH in the Blood in Normal and Nephritic Rabbits before and after Fasting.

Rabbit No.	Date.	Body weight.	Blood. Sugar.	Blood. CO_2 capacity in plasma.	Blood. pH	Remarks.
		gm.	*per cent*			
	Dec. 26, 1917, 2 p.m.	2,580	0.128	47.2	7.3	No evidence of kidney
21	" 27, 1917, 8 a.m.		0.111	56.5	7.45	disease, no glycosuria.
	4 p.m.		0.111	53.5	7.35	
	Dec. 31, 1917, 2 p.m.	1,560		50.7	7.4	No evidence in urine of
23	Jan. 1, 1918, 8 a.m.		0.122	52.5	7.5	any disease.
	4 p.m.		0.096	53.8	7.35	
	Dec. 30, 1917, 2 p.m.	1,950	0.143	36.4	7.28	Heavy albuminuria and
22	" 31, 1917, 8 a.m.		0.139	42.0	7.4	casts, no glycosuria.
	4 p.m.		0.119	34.0	7.25	
	Jan. 2, 1918, 2 p.m.	1,760	0.089	42.9	7.2	Heavy albuminuria, no
24	" 3, 1918, 8 a.m.		0.100	44.0		glycosuria.
	4 p.m.		0.099	43.2	7.4	
	Dec. 24, 1917, 2 p.m.	2,780	0.133	51.9	7.4	Moderate albuminuria
20	" 25, 1917, 8 a.m.		0.133	31.9	7.28	and casts.
	4 p.m.		0.122	32.0	7.25	
	Dec. 28, 1917, 2 p.m.	2,620	0.135	46.9	7.3	From Dec. 28 to 29 gave
20	" 29, 1917, 2 "		0.130	51.6	7.45	oats and water freely.
	" 30, 1917, 2 "		0.106	42.2	7.35	Dec. 29 to 30, all fasted.

the following day—over 24 hours. The second blood was drawn the following morning, and the third sample in the afternoon. The urine of all rabbits was tested for albumin, casts, and sugar.

In Table I Rabbits 21 and 23 have no evidences of kidney disease. No appreciable change in the hydrogen ion concen-

TABLE II.

Variation of CO_2 Capacity and pH in Blood before and after the Administration of Guanidine.

Rabbit No.	Date.	Body weight.	Blood.			Remarks.
			Sugar.	CO_2 capacity in plasma.	pH	
		gm.	*per cent*			
12	Dec. 8, 1917, 8 a.m.	1,800	0.110	43.3	7.4	0.25 gm. of guanidine per kilo at 8 a.m. Symptoms 1 hr. after injection.
	2 p.m.		0.190	—	—	
	4 "		0.195	—	—	
	7 "		0.143	22.5	7.2	
13	Dec. 10, 1917, 8 a.m.	1,800	0.125	45.0	7.45	0.2 gm. of guanidine per kilo at 8 a.m. Symptoms 10 min. after injection.
	3 p.m.		0.075	—	—	
	4 "		0.073	23.9	7.25	
14	Dec. 12, 1917, 9 a.m.		0.111	45.1	7.4	0.2 gm. of guanidine per kilo at 8 a.m.
	4 p.m.		0.105	44.0	7.45	
	" 13, 1917, 8 a.m.	2,620	0.105	55.5	7.5	Symptoms a few min. after 1st injection.
	4 p.m.		0.154	22.1	7.1	
	" 14, 1917, 2.30 "		0.100	20.4	7.1	0.12 gm. of guanidine per kilo at 8 a.m.
	4 "		0.087	17.6	7.0	
15	Dec. 14, 1917, 2 p.m.		0.111	48.1	7.35	0.2 gm. of guanidine per kilo at 10 a.m.
	" 15, 1917, 8 a.m.	1,940	0.095	44.1	7.35	No appreciable symptoms after 1st injection.
	3 p.m.		0.195	44.3	7.33	
	" 16, 1917, 9 a.m.		0.133	54.5	7.5	0.15 gm. of guanidine per kilo at 9 a.m. Symptoms 10 min. after 2nd injection.
	3 p.m.		0.077	35.3	7.25	
	5 "		0.064	29.4	7.2	
16	Dec. 21, 1917, 3 p.m.	2,120	0.111	49.0	7.4	0.2 gm. of guanidine per kilo at 8 a.m.
	" 22, 1917, 8 a.m.		0.100	55.0	7.4	Symptoms 10 min. after 1st injection.
	2 p.m.		0.133	33.9	7.15	
	4 "		0.118	31.9	7.1	
	" 23, 1917, 8 a.m.		0.100	24.8	7.2	0.1 gm. of guanidine per kilo at 8 a.m.
	4 p.m.		0.050	19.9	7.05	
21	Jan. 3, 1918, 8 a.m.	2,460	0.125	54.5	7.4	0.2 gm. of guanidine per kilo at 8 a.m. Symptoms ½ hr. after injection.
	4 p.m.		0.080	33.9	7.2	
	" 4, 1918, 8 a.m.		0.071	36.1	7.3	

TABLE II—*Concluded.*

Rabbit No.	Date.	Body weight.	Blood.			Remarks.
			Sugar.	CO_2 capacity in plasma.	pH	
		gm.	*per cent*			
22	Jan. 5, 1918, 8 a.m.	1,960	0.125	54.8	7.5	0.2 gm. of guanidine per kilo at 8 a.m. Severe symptoms 10 min. after injection. Albumin and casts in urine.
	4 p.m.		0.159	40.2	7.3	
	" 6, 1918, 8 a.m.		0.111	31.9	7.15	
20	Jan. 5, 1918, 9 a.m.	2,550	0.130	47.1	7.4	0.2 gm. of guanidine per kilo at 9 a.m. Symptoms 1½ hr. after injection. Albumin and casts in urine.
	4 p.m.		0.089	31.9	7.2	
	" 6, 1918, 9 a.m.		0.082	22.5	7.15	

tration or CO_2-combining power was observed in 24 hours under the fasting conditions. The other rabbits had albumin and some of them casts, and the blood had a tendency to become more acid in the last specimen after fasting. In all cases there was a slight inclination toward acidosis.

Table II demonstrates that after the injection of the sublethal dose of guanidine hydrochloride, marked acidosis appeared in every rabbit, as shown by the increased hydrogen ion concentration in the blood and the decrease in the alkaline reserve.

In some cases, Nos. 14 and 16 which had been injected twice, there was marked acidosis. No. 15 showed no appreciable symptoms of tetany following the first injection of the drug and no acidosis occurred. A second injection was made and this time symptoms appeared 1 hour after the administration and later distinct acidosis followed. All the other rabbits manifested symptoms of tetany after the first injection and acidosis developed in all cases. From these observations it appears that acidosis may appear only in those cases which show symptoms of tetany. In some cases two or three samples of blood were drawn at 7 or 18 hour intervals, giving oats and water freely, and there was no evidence of acidosis. The administration of guanidine produces

marked acidosis and, moreover, if the results on Nos. 22 and 20, which have albuminuria, are compared to those of Table I in which no drug was administered, there is no more question that the condition of tetany produces a markedly high degree of acidosis.

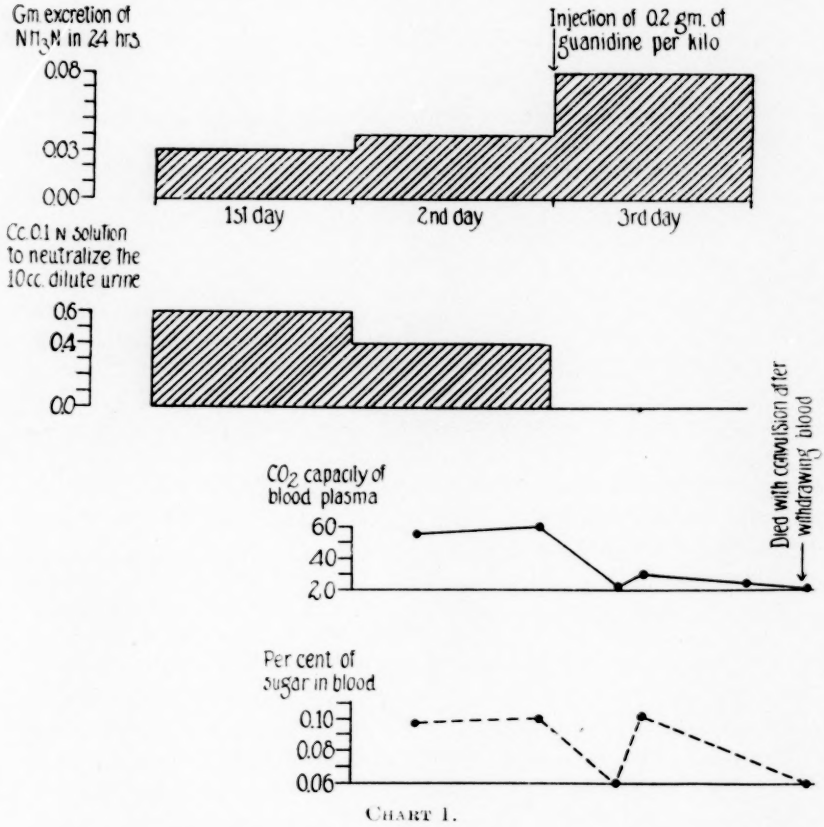

CHART 1.

Relationship of Hypoglycemia to Acidosis.

It was reported in the preceding paper (14) that the hypoglycemia occurring several hours after guanidine injection is also manifested after parathyroidectomy and it may be considered from the above evidence that the hypoglycemia thus produced is the secondary phenomenon of guanidine poisoning. The estimation

of blood sugar was carried on in the present investigation. In cases where guanidine was not administered the blood sugar remained normal but where guanidine was given the hypoglycemia was produced several hours after the injection in most cases. The specimens with the low percentage of sugar show a high acidosis. However, the hypoglycemia and the acidosis are not strictly parallel, for some cases show a high acidosis and no hypoglycemia. Hence we may consider that certain other factors might play a part in this phenomenon. To make clear the intimate relation which exists between acidosis and hypoglycemia, the urine and blood of one rabbit were examined at the same time before and after guanidine administration. After the injection of the drug the ammonia in the urine became extremely high and also there appeared a high ratio of ammonia to total nitrogen. At the same time the urine became neutral and there was a decrease in the hydrogen ion concentration. The blood showed a marked decrease in alkaline reserve and a high hydrogen ion concentration. The chart shows the relationships between the results on the urine and blood.

SUMMARY.

1. The injection of guanidine produces a marked increase in hydrogen ion concentration and a decrease in the CO_2 capacity of the blood following the manifestation of symptoms of tetany.

2. The hypoglycemia which appears after the administration of guanidine is manifested only after the acidosis becomes severe, and there is no parallelism between the severity of acidosis and hypoglycemia.

The injection of large doses of guanidine or of several small doses into rabbits produces increased elimination of ammonia, and the ratio of ammonia nitrogen to the total nitrogen is increased. At the same time there is a decrease of acid excretion and a lowering of the hydrogen ion concentration of the urine (14). These phenomena also appear in tetania parathyreopriva. A high degree of acidosis is produced following the symptoms of tetany by the injection of guanidine. From the above facts we are inclined to believe that the excretion of the excess of NH_3 in the urine in tetany is a protective mechanism for the liver to neutralize the acid which is produced by the excess muscular activity,

rather than the depression of the function of the liver as suggested by Carlson and Jacobson (1), and in the course of this investigation we noted that the more the excitation the greater the elimination of ammonia.

CONCLUSION.

The metabolic effects induced by the administration of guanidine hydrochloride to the animal body resemble strikingly those of tetania parathyreopriva, such as excess of ammonia, decrease of acid elimination in the urine, and the development of acidosis in the body. These facts further strengthen the assumption that guanidine plays an important rôle in the development of tetany.

The writer is indebted to Professor F. P. Underhill for his advice throughout the investigation.

BIBLIOGRAPHY.

1. Carlson, A. J., and Jacobson, C., *Am. J. Physiol.*, 1909–10, xxv, 403.
2. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *J. Biol. Chem.*, 1915, xxiii, 123.
3. Wilson, D. W., Stearns, T., and Thurlow, M. DeG., *J. Biol. Chem.*, 1915, xxiii, 89.
4. Underhill, F. P., and Saiki, T., *J. Biol. Chem.*, 1908–09, v, 225.
5. Cooke, J. V., *J. Exp. Med.*, 1911, xiii, 439.
6. Greenwald, I., *Am. J. Physiol.*, 1911, xxviii, 103.
7. Engelman, G. J., *Arch. Anat. u. Physiol., Anat. Abt.*, 1871, 14.
8. Klug, F., and Olsavszky, V., *Arch. ges. Physiol.*, 1893, liv, 21.
9. Dunlop, J. C., Paton, D. N., Stockman, R., and Maccadam, I., *J. Physiol.*, 1897-98, xxii, 68.
10. Greenwald, *J. Biol. Chem.*, 1913, xiv, 363.
11. Greenwald, *J. Biol. Chem.*, 1913, xiv, 369.
12. Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.
13. Henderson, L. J., *J. Biol. Chem.*, 1911, ix, 403.
14. Watanabe, C. K., *J. Biol. Chem.*, 1918, xxxiv, 51.
15. Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.
16. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
17. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
18. Sellards, A. W., *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 289.
19. Palmer, W. W., and Henderson, L. J., *Arch. Int. Med.*, 1913, xii, 153.
20. Goto, K., *J. Exp. Med.*, 1917, xxv, 693.

STUDIES IN THE METABOLIC CHANGES INDUCED BY ADMINISTRATION OF GUANIDINE BASES.

IV. THE INFLUENCE OF THE ADMINISTRATION OF CALCIUM UPON BLOOD SUGAR CONTENT IN RABBITS WITH GUANIDINE HYPOGLYCEMIA.

By C. K. WATANABE.

(From the Laboratory of Pathological Chemistry, School of Medicine, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

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It has been demonstrated that the hypoglycemia manifested after thyreoparathyroidectomy (1) can also be produced by the injection of guanidine hydrochloride (2). In a recent communication (3) it was pointed out that the hypoglycemia after injection of guanidine has an intimate connection with the development of acidosis in the animal body which is probably caused by the violent muscular activity. It was pointed out also that there is no strict parallelism between the acidosis and hypoglycemia, for in some cases there is a high acidosis without hypoglycemia. Thus we may consider that other factors play a part in this phenomenon.

Underhill and Blatherwick (4) demonstrated that the hypoglycemia and the condition of tetany produced by thyreoparathyroidectomy can be abolished temporarily by injecting calcium lactate, which fact seems to point to a calcium deficiency as a factor in tetany. The repeated injection of calcium lactate maintained the normal level of blood sugar content, while cessation of calcium treatment led to a state of continued hypoglycemia. They deduced from the above phenomena that, in tetany, calcium plays an important part in maintaining the equilibrium of the sugar-regulating mechanism. When there is a sufficiency of calcium the blood sugar level is normal, while a deficiency of calcium is correlated with low blood sugar content.

From the above facts it seems possible that the low blood sugar content, induced by injection of guanidine, may be raised to the normal level by the injection of calcium. The present investigation has been instituted to test this point.

Methods.

Rabbits were used and the blood sugar and CO_2 capacity of the plasma were estimated with the methods mentioned in the previous papers. Calcium lactate was injected subcutaneously after the production of guanidine hypoglycemia, and the sugar content and CO_2 capacity were compared before and after calcium injection.

The sublethal dose of guanidine hydrochloride was given to every rabbit, though it was divided and given at intervals of several hours in order to prolong the period of hypoglycemia which precedes the terminal convulsion. In most cases the first injection was made on the day previous to the one on which calcium was given. One specimen of blood was drawn before guanidine was injected to serve as a normal standard. The second injection of a smaller dose of the drug was given the following morning. The hypoglycemia appeared several hours after the second injection, in most cases; then a 5 per cent solution of calcium lactate was given subcutaneously.

DISCUSSION.

In four out of eight experiments there was no recovery of the normal blood sugar level. In two cases (Nos. 3 and 4) blood sugar content remained normal throughout the experiment. Guanidine hypoglycemia was not in evidence and the injection of calcium was without significant influence upon the level of blood sugar.

In two other instances (Nos. 8 and 10) blood sugar content was apparently restored to normal or even above by calcium injection. Both rabbits had had previous injections of the drug. In Paper III it was stated that the second or third injection produced hypoglycemia only after a larger dose of the drug. Under these conditions all the animals die. In Rabbit 10, a very slight degree of hypoglycemia was manifested, and No. 8 showed no hypoglycemia. Both of these animals were in better condition than the other cases until death. After calcium administration the blood sugar content increased. Hence we may remark that previous injections of guanidine so alter the metabolism that no hypoglycemia occurs in spite of high acidosis. All the rabbits showing guanidine hypoglycemia are weak and sensitive and recover with difficulty. Moreover, the manipulation of taking

TABLE I.

Rabbit 8 (2,000 gm).

Experimental day.	Blood sugar.	Plasma CO_2 capacity.	Remarks.
	per cent		
1st day, 3 p.m.	0.097	49.7	
2nd " 10 a.m.			0.2 gm. guanidine hydrochloride per kilo subcutaneously at 10 a.m. ½ hr. later symptoms appear.
3rd " 9 "	0.111		0.05 gm. guanidine hydrochloride per kilo subcutaneously at 8.30 a.m. Animal sensitive.
11 "	0.097		Symptoms reappear at 11 a.m. after 2nd injection.
2 p.m.		25.2	
2.50 "	0.100		3 cc. 5 per cent calcium lactate per kilo subcutaneously at 2.50 p.m. Animal sensitive.
3.50 "	0.125	25.5	From 4.30 p.m. condition of animal good.
7 "	0.175	19.0	During withdrawal of blood animal died with convulsion.

blood from the jugular vein and the large quantity demanded (7 or 8 cc. several times a day) result in a depressed condition. The rabbits died, in most cases, a few hours after calcium injection.

In most cases, administration of calcium had no effect on the CO_2 capacity of the plasma.

This is true even in the case in which the blood sugar increased after the injection of calcium. Rabbits 2 and 6 had no high acidosis but marked hypoglycemia, while No. 8 shows high acidosis but no hypoglycemia.

In most cases, injection of calcium solution improved the condition of the animal and diminished the excitation, though the life of the animal was not prolonged. Beside the above experiments two rabbits, which had had guanidine administered, were injected with calcium at the time of the most severe symptoms—1 or 2 hours after guanidine injection—but the calcium had no effect on these symptoms.

Although the above experiments failed to demonstrate the restoration of the blood sugar level by calcium injection in condition of guanidine tetany, the condition may be different from that of parathyroid tetany where any increase in guanidine in

TABLE II.

Rabbit 6 (2,040 gm.).

Experimental day.	Blood sugar.	Plasma CO_2 capacity.	Remarks.
	per cent		
1st day, 3 p.m.	0.100	57.2	
2nd " 10 a.m.			0.2 gm. guanidine hydrochloride per kilo at 10 a.m. From 10.20 a.m. dyspnea, twitching of head, then depression. At 4 p.m. diarrhea, disturbance of balance, twitching of muscles.
3rd day, 10.20 "	0.092		0.05 gm. guanidine hydrochloride per kilo at 9 a.m. 11 a.m. depression and twitching of head.
1.30 p.m.	0.072		
2 "	0.064	38.5	3 cc. 5 per cent calcium lactate solution per kilo subcutaneously at 2.10 p.m. From 3.30 p.m. condition of animal better.
4 "	0.056	37.6	Depression, otherwise normal.
7 "	0.044	31.5	7.10 p.m. convulsion, opisthotonus, and death.

the body, if the guanidine is the only cause of tetany, may be more gradual than under the present experimental conditions.

SUMMARY.

The present experiments confirm previous observations that there is a correlation between the hypoglycemia and acidosis induced by guanidine injection. It is also probable that factors other than the conditions of acidosis play a rôle in the disturbance of equilibrium of the sugar-regulating mechanism thus manifested.

In animals with guanidine hypoglycemia injection of calcium lactate failed to restore the blood sugar content to normal.

Unlike the tetany induced by thyreoparathyroidectomy that involved by guanidine is not abolished by calcium administration.

BIBLIOGRAPHY.

1. Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.
2. Watanabe, C. K., *J. Biol. Chem.*, 1918, xxxiii, 253.
3. Watanabe, *J. Biol. Chem.*, 1918, xxxiv, 65.
4. Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 119.

THE STOICHIOMETRICAL CHARACTER OF THE ACTION OF NEUTRAL SALTS UPON THE SWELLING OF GELATIN.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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I.

It is generally believed that the reactions of colloids do not obey the stoichiometric laws of general chemistry. This viewpoint is illustrated clearly in the discussion of the action of neutral salts upon such physical qualities of proteins, as the swelling power of gelatin. The colloid chemists state that acetates, sulfates, tartrates, and citrates influence the swelling in the opposite sense from chlorides, bromides, and nitrates, the latter causing more swelling of the gelatin than distilled water, while the former cause a shrinking of the gelatin. Moreover, they claim that the relative efficiency of the anions of the same group varies in a definite order. The influence of the cations is stated to be negligible. In a previous paper the writer has already reported facts showing clearly that the effect of neutral salts upon the swelling of gelatin is of a stoichiometrical character.[1] Thus it was shown that all the univalent cations of neutral salts, Li, Na, K, and NH_4, influence the swelling of gelatin at exactly the same concentration no matter what the nature of the anion; and the writer had also proved that only an unsuitable method was responsible for the erroneous statements of the colloid chemists concerning the influence of salts upon the swelling of gelatin.

The source of error into which the colloid chemists have fallen in this case is not difficult to understand. It happens that an adequate chemical reaction between neutral salts and proteins

[1] Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531.

occurs only when the salt is added in excess, and it also happens that a number of the physical qualities of the new salt-protein compound only become apparent if the excess of the salt which acted upon the protein is afterwards washed away. In most previous experiments the effect of the salt upon the protein was tested in the presence of the salt; so that when little salt was used no or too small an effect on the protein was produced and when too much salt was used the salt present prevented the manifestation of the new qualities of the salt-protein product. Thus in Hofmeister's experiments[2] the swelling of a block of gelatin was measured in the presence of an excess of salt and he measured in reality the resultant of two opposite effects; namely, the mass action of the salt upon the protein and the inhibiting effect of the salt upon the swelling of the new salt-protein compound. It is no wonder that with such methods no stoichiometrical results were obtained.

Our method consisted in perfusing 2 gm. of finely powdered, non-bleached gelatin of equal size of grains (going through sieve size 50 but not through 60) twice with 25 cc. M/8 NaCl. It was assumed that with this excess of salt a certain percentage of the gelatin molecules would react with the NaCl and form a new compound. The gelatin molecules being amphoteric but stronger as acid than as base should form with the salt a compound which we will symbolize as Na-G (sodium gelatinate). When we remove the salt by perfusing the powder four times with 25 cc. of H_2O the Na-G formed is supposed to remain after the salt solution is washed away. Hence it is the additional swelling of this sodium gelatinate which served for the experiments in our previously published notes.

This compound dissociates electrolytically into $\overset{-}{G} + \overset{+}{Na}$, as was indicated by the fact that in the electrical field gelatin treated with NaCl, and then washed with H_2O, migrates exclusively to the anode (as does the gelatin not treated with salt, which is a stronger acid than base). This leaves the question open whether or not the Cl combines also with the gelatin or whether the reaction is only between Na and gelatin; in other words, Which of the two following possible reactions takes place?

[2] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1891. xxviii, 210.

1. Gelatin$\begin{cases}\text{NH}_2\text{H}\,.\,\text{OH}\\ \text{COOH}\end{cases}$ + NaCl = gelatin$\begin{cases}\text{NH}_2\text{HCl}\\ \text{COONa}\end{cases}$ + H_2O

2. Gelatin$\begin{cases}\text{NH}_2\text{HOH}\\ \text{COOH}\end{cases}$ + NaCl = gelatin$\begin{cases}\text{NH}_2\text{HOH}\\ \text{COONa}\end{cases}$ + HCl

If the second type of reaction takes place, free acid, HCl, should appear in the supernatant salt solution. This was apparently not the case in our experiments. It should, however, be borne in mind that at least part of any free acid formed would combine with the gelatin and this combination between HCl and gelatin might occur not only in molecules of the type gelatin$\begin{cases}\text{NH}_2\text{HOH}\\ \text{COONa}\end{cases}$ but also in the molecules not in combination with Na; namely, gelatin$\begin{cases}\text{NH}_2\text{HOH}\\ \text{COOH}\end{cases}$ It is very probable that the reaction between gelatin and NaCl in our experiment is not complete; *i.e.*, does not transform all the gelatin molecules into Na gelatinate. If, therefore, part of the acid is caught by the gelatin, the fact that the reaction of the supernatant salt solution does not become markedly acid does not speak against the assumption that the reaction which occurs is the second reaction, which results in the formation of a sodium gelatinate. The decision of the question is not essential for our present purpose. We may state that when we treat gelatin with an excess of NaCl a compound is formed which dissociates electrolytically into Na^+ and a negatively charged gelatin ion. If this gelatin ion contains also Cl the Cl is held in non-dissociable or less dissociable bondage; so that for all practical purposes we are dealing with a compound which we may call sodium gelatinate and which we will symbolize as Na-G.

Non-treated gelatin of the type gelatin$\begin{cases}\text{NH}_2\text{H.OH}\\ \text{COOH}\end{cases}$ swells in distilled water, but the swelling soon reaches its maximum. Sodium gelatinate swells also when perfused with an M/8 NaCl solution and reaches a maximum (which is lower than that of non-treated gelatin in H_2O). This swelling is the *initial* swelling which has nothing or little to do with the effect of the salt and which will be

disregarded by us. If we remove, however, the excessive salt by perfusing the powder repeatedly with a NaCl solution weaker than M/8 we find that below a definite concentration (M/64) of NaCl an *additional* swelling of the gelatin takes place, which becomes the greater the greater the dilution of the washing solution. This *additional* swelling which is the true effect of the salt upon the swelling of the gelatin we assumed to be due to the existence of the negative gelatin ion. That the gelatin ion has a negative charge in this case has nothing to do with the phenomenon of additional swelling, since positively charged gelatin ions (*e.g.*, gelatin treated with acid) also give rise to an additional swelling. The assumption that the ionization of the gelatin is responsible for the *additional* swelling is not necessary for the proof of the stoichiometrical character of the influence of neutral salts on the swelling, though everything speaks in favor of this assumption.

The fact of importance for us is the following: An excess of NaCl is necessary to cause the formation of Na gelatinate and the *additional* swelling only begins to appear if the excess of the salt is removed and the M/8 NaCl solution is replaced by a M/64 NaCl solution. If the M/8 NaCl is replaced by a still weaker NaCl solution the additional swelling becomes still greater. *The stoichiometrical character of the inhibiting influence of neutral salts upon the additional swelling is revealed in the following facts.*

1. All neutral sodium salts with monovalent anion have under the conditions of the experiment the same limiting molecular concentration for the beginning of the additional swelling, namely M/64 (Table I, Group I).

2. All neutral sodium salts with bivalent anion have the same limiting molecular concentration for the beginning of the additional swelling which is M/128—exactly half as large as that for the salts with monovalent anion. Since a M/128 solution of Na_2SO_4 has approximately as many sodium ions in solution as a M/64 NaCl solution, we can say that in all neutral solutions of a sodium salt with monovalent or bivalent anion the limiting concentration for the additional swelling is determined by the concentration of Na ions in the solution which is M/64 regardless of the anion (Table I, Group II).

3. The limiting concentration for the additional swelling remains the same when the salts treated first with M/8 NaCl are after-

wards perfused with neutral solutions of salts of Li, K, and NH_4 with monovalent or bivalent anion (Table I, Groups I and II).

4. Non-electrolytes, like cane sugar, glycerol, alcohol, have no such limiting effect upon the additional swelling in concentrations of 2 M and below, but behave like distilled water (Table I, Group V).

These four facts are only comprehensible on the assumption that the additional swelling of gelatin depends upon the formation of well defined metal gelatinates in which the metal ions, Li, Na, K, and NH_4, can replace each other in the same stoichiometric way as in the case of simple inorganic salts. The fact that the nature of both bivalent and univalent anions and univalent cations of the salt are of little if any importance fits well with the assumption that it is the ionization of the metal gelatinate which determines the additional swelling (Table I).

In order to understand the figures it should be stated that the perfusion of gelatin occurred in cylindrical funnels of the same diameter so that the increase in the height of the cylinder, expressed in the table in millimeters, was the measure of the swelling. The measurements were usually made after 24 hours to allow all the water which was not held by the gelatin or in the minute capillary spaces between the granules to run off.[3, 1]

The perfusion of the 2 gm. of gelatin with 50 cc. M/8 NaCl causes a considerable initial swelling which does not increase when the perfusion with M/8 NaCl is repeated. When, however, for the subsequent perfusion (following that with M/8 NaCl) a weaker solution, namely M/64 or M/128 NaCl, is used, *additional* swelling takes place and this *additional* swelling is the true effect of the previous salt treatment upon the swelling of gelatin. As long as this additional swelling is less than 3 or 4 mm. in the height of the cylindrical mass of gelatin it may be disregarded since this is within the limits of unavoidable variation, due mainly to the formation of air bubbles in the mass and the error in measuring the height of the cylinder. The real additional swelling begins with an increase of 5 or 6 mm. in the height of the cylindrical mass of gelatin. The figures in Table I give this increase in the height of the cylindrical mass of gelatin in millimeters. The

[3] Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

TABLE I.

Action of Neutral Salts upon the Additional Swelling of Sodium Gelatinate Produced by the Action of NaCl upon Gelatin.

Additional swelling of column of gelatin perfused twice with 25 cc. M/8 NaCl (to produce sodium gelatinate) and three times with 25 cc. of the following solutions.

Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
		mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
I. Univalent cation, univalent anion.	NaCl	0	0	2.5	**6**	15	21	33.5	42	48			54
	NaBr	0	2.0	2.0	**5**	12.5	17.5	27	43	42	46	53	52.5
	$NaNO_3$	1	?	4	**7**	12.5	19.5	29.5	39	43.5	51	49	54
	Na acetate	1.5	1.5	3	**6.5**	15	22	31	38	45.5	44	48	55
	NaCNS		3.5	4	**7**	12	21	31	40	46	53	59	60
	LiCl	1	1.5	3.5	**6**	10.5	15	24.5	34.5	44.5	52	53.5	57.5
	KCl	1	2	2.5	**6.5**	11.5	16	24	33	38.5	44	46.5	53.5
	NH_4Cl	2	2	2	**5**	13	23	22.5	39.5	48	47		56
II. Univalent cation, bivalent anion.	Na_2SO_4	1	1.5	2.5	3.5	**8**	14	22.5	30	39	47	55	54
	Na_2 oxalate	2	2	2	3.5	**6.5**	14.5	24	29	35	46	50	56
	Na_2 tartrate	2	2	2	3	**5.5**	13.5	23	32.5	43	46	52.5	56.5
	Na_2 malate	0	1	1	2.5	**5**	13	20	31	35	39	43	51
	Na_2 succinate		1	1	1.5	**6**	14.5	21	28.5	35	46	47.5	51.5
	Li_2SO_4	3	0	2	2.5	3.0	**10**	19.5	30.5	45.5	51	51	60
	K_2SO_4	1	1.5	0.5	3.0	**6.5**	11	20	29	40.5	42	50.5	54.5
	$(NH_4)_2SO_4$	1.5	1.5	2	3	**6**	12	18	26	42	50	45	53
	K_2 oxalate	1	1	1.5	3.5	**7.5**	17	22.5	25	33.5	43	46.5	55
	$(NH_4)_2$ oxalate	0	1	1	2.5	**5**	11.5	22.5	27	36.5	48.5	49	54
	K_2 tartrate	0	0	1.5	1.5	**7**	13	19	28	37	42	46	53
	$(NH_4)_2$ tartrate	2	1.5	2	5?	**7**	16.5	22	35.5	43.5	49.5	52	55

III. Bivalent cation, univalent anion.	$MgCl_2$	0	0	0	0	0	4	13	26	42	49	53	52
	$CaCl_2$	0	0	0	0	0	1.5	8	24	39	48	51	47
	$SrCl_2$	0	0	0	0	0	1	8	18	36	43	52	52
	$BaCl_2$	0	0	0	0	0	1	10	21	40	45	51	50
IV. Bivalent cation, bivalent anion.	$MgSO_4$	1.5	0	0	−1	0	1	7.5	22.5	38	48	55	63
	$CaSO_4$					− 3.5	− 2.5	+ 8.5	18.5	37.5	43	47	55
V. Non-electrolytes.	Glycerol	43	42	42	40	41	41	41	43				42
	Cane sugar	51	51	50	50	51	51	51					48
	Ethyl alcohol	48.5	49.5	49.5	49.5	47	47.5	47					50.5

reader will notice that the increase between 5 and 7 mm. lies at M/64 for all salts of Group I (type NaCl), while it lies at M/128 for all salts of Group II (type Na_2SO_4) regardless of the nature of the anion. This is the most fundamental of our results whereby the fact is established that we are dealing with simple chemical substitution phenomena.

Perfusion of Na gelatin with M/128 solutions of the salts of Group I (type NaCl) causes an additional swelling of from 10 to 15 mm. in the height of the cylindrical mass of gelatin; the same quantitative effect is found for concentrations of M/256 for salts of Group II (type Na_2SO_4). All through the table it is obvious that the salts of the type Na_2SO_4 give the same figures for additional swelling when their molecular concentration is one-half of that for Group I, type NaCl. This is true regardless of the nature of the anion or cation, as long as the latter belongs to the alkali group including NH_4.

When we perfuse the gelatin (previously treated with M/8 NaCl) with solutions of non-electrolytes (sugar, glycerol, alcohol) of different concentration we get the same effect as when we use distilled water. This is one of the reasons why we are inclined to assume that the limiting concentration for the action of neutral salts upon swelling is that concentration which allows so many of the sodium gelatinate molecules to dissociate electrolytically that the additional swelling can begin. If our assumption is correct, the figures given in Table I might be utilized to calculate roughly the degree of electrolytic dissociation of the Na gelatin molecules in the solution of a salt of a given concentration, by taking the dissociation in H_2O as 100 per cent.

The reader will notice that we do not utilize the absolute amount of swelling as a criterion for our conclusions, but use only equal swelling in different concentrations of salt. The absolute swelling cannot be estimated by our figures since they include not only the water held inside the gelatin grains (the true swelling) but also the water held in the fine capillary spaces between the powdered granules which, however, may be only a comparatively small quantity.

It seemed desirable to see whether we could determine the limiting concentration for the swelling caused by neutral salts of Type I (NaCl) and Type II (Na_2SO_4) a little more accurately.

For this purpose 2 gm. of powdered gelatin were perfused twice with 25 cc. M/8 NaCl and then with solutions from between 16 M/512 to M/512 of different salts of Types I and II. In this case we found again that the molecular concentrations of salts of Type I (NaCl) which just permit swelling are twice as great as those for salts of Type II (Na_2SO_4), and that the limiting concentrations are approximately between 3/512 and 4/512 for Salts II (Na_2SO_4)and between 3/256 and 4/256 for Salts I (NaCl) (Table II).

TABLE II.

More accurate determinations of the limiting concentration of different salt solutions upon the additional swelling of column of sodium gelatinate.

	8M/256	7M/256	6M/256	5M/256	4M/256	3M/256	5M/512	4M/512	3M/512	2M/512	M/512
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
NaCl	1.5	1	2	3.5	5	7		11.5		21.5	
$NaNO_3$	1.5	3.5	2	2	3.5	8.5		13		23	
NaBr	2.5	2	3.5	3.5	5.5	8		14		24	
NaCNS	2	3	3	3.5	4	9		14		19.5	
LiCl	1.5	2	2	2	1.5?	5		10		18	
K acetate	2	1	4	4.5	3	10.5		16		26.5	
NH_4Br	1	0	2.5	3	4.5	7		14		22	
$(NH_4)_2SO_4$	1	1	0	0	1	1	2.5	4.5	8	13	21
Na_2 malate	1		0	1	0	0.5	1	4	5.5	10	22.5
Na_2 tartrate	1	1	1	1	0	1.5	3.5	5.5	9	16.5	22.5
Na_2 oxalate	0	1	2	1	1	2	5.5	5.5	9	12	
K_2SO_4					1.5	1.5	4	5	3	13	

From these facts it follows that only the cation inhibits the swelling in this case and that univalent cations, Li, Na, K, NH_4, all act in exactly the same way in the same concentration no matter what the nature of the anion is. *We can, therefore, use the limiting concentration of a neutral salt for the additional swelling of sodium gelatinate as a rough method for determining the molecular concentration of the salt and for this reason we are entitled to state that the action of the neutral salts upon the swelling of gelatin is of a strictly stoichiometrical characters.*

In all the experiments thus far reported we always used the same ion-gelatin compound; namely, Na gelatinate prepared by

treating the gelatin with M/8 NaCl. We have stated in a previous paper that we get the same results when we first treat the gelatin with some other neutral salt; *e.g.*, LiCl.

The effect of the neutral salts of the alkaline earth metals requires a special discussion. We have found that the salts of Mg, Ca, Sr, and Ba do not cause an additional swelling, and we are forced to the conclusion that such salts form either non-ionizable gelatin compounds or compounds of a different type from those which originate when salts of the alkali metals combine with gelatin. Pfeiffer and von Modelski[4] have shown that Ca forms with amino-acids and betaines compounds containing two or three atoms of amino-acid in the molecule; namely,

$$Ca\begin{matrix}ACl\\ \\ ACl\end{matrix} \quad \text{as well as} \quad Ca\begin{matrix}ACl\\ \\ AACl\end{matrix}$$

The idea suggests itself that similar products originate when the metals of the alkaline earths combine with proteins like gelatin and that these larger compounds (of the type calcium gelatinate) do *not* cause any additional swelling. We will represent such compounds under the symbol $Ca\begin{matrix}gelatin\\ \\ gelatin\end{matrix}$ while the compound really formed may be of a more complicated or different nature than represented by our symbol and may also contain Cl. If it is the ionized gelatin which causes the additional swelling it follows that such compounds as calcium gelatinate do not dissociate electrolytically or if they do so their ionization will result in the formation of a polymerized or otherwise more complex gelatin anion, which for some unexplained reason is not capable of swelling.

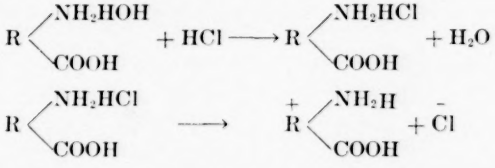
That the neutral salts $MgCl_2$, $MgSO_4$, $CaCl_2$, $CaSO_4$, $SrCl_2$, and $BaCl_2$, react with gelatin under the formation of definite metal gelatinates and that these compounds determine the negative influence upon swelling is indicated by the fact that the limiting concentration is identical for all these salts; namely, about M/512 (Table I, Groups III and IV). This means that when we first perfuse gelatin with M/8 NaCl to form sodium gelatinate and then wash the NaCl away with solutions of salts of the alkaline

[4] Pfeiffer, P., and von Modelski, J., *Z. physiol. Chem.*, 1912, lxxxi, 329; 1913, lxxxv, 1.

earth metals, the additional swelling begins to appear when the concentration of the $CaCl_2$, etc., is about M/512 or below. The fact that $CaCl_2$ and $CaSO_4$, and $MgCl_2$ and $MgSO_4$ act alike confirms again the stoichiometrical character of the action of these salts on the gelatin.

II.

Hardy made the discovery that proteins when treated with acid migrate to the cathode and when treated with a base to the anode, and this fact was first interpreted as a "colloidal" phenomenon due to the "adsorption" of the H or HO ion by the protein[5] or to the "transfer of the electric charges" of these ions to the protein. The writer pointed out that no such explanation was needed and that the phenomenon was a simple case of electrolytic dissociation due to the fact that the protein molecule is an amphoteric electrolyte, which in the presence of acid must dissociate as a base and become a cation, while in the presence of base it must dissociate as an acid and become an anion.[6] This chemical view has since been generally accepted but with an important alteration suggested by Hardy that when acid is added to a protein a salt is formed which dissociates into the anion of the acid added and a positive protein ion.[7]



Therefore, if we treat gelatin with acid, *e.g.* HCl, a salt (gelatin chloride) is formed which dissociates into a positively charged gelatin ion and a negatively charged chlorion. When a 1 per cent solution is made of gelatin previously treated with HCl the gelatin no longer migrates to the anode but to the cathode.

This new compound, gelatin chloride, which dissociates into

[5] The protein ion was supposed to be charged positively by the adsorbed H ion and negatively by the adsorbed HO ion.

[6] Loeb, *University of California Publications, Physiology*, 1902–04, i. 149.

[7] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

gelatin$^+$ and Cl^- gives us an ideal chance to test our stoichiometric theory. If this theory is right we should expect that if we first treat powdered gelatin with dilute HCl, and follow this with three or four perfusions with various concentrations of neutral salts, we should expect the following results:

1. Neutral salts of the type NaCl (*e.g.* univalent anion, univalent cation) should permit additional swelling of gelatin chloride at the same degree of dilution which under the conditions of our experiments was again M/64.

2. Neutral salts of the type $CaCl_2$ (*i.e.* alkaline earth metals and univalent anion) should cause additional swelling of gelatin chloride in molecular concentrations exactly twice as dilute as salts of the type NaCl; namely, from M/128 down. The reader will notice that $CaCl_2$ should act upon gelatin chloride exactly as salts of the type Na_2SO_4 acted upon sodium gelatinate in the previous experiments reported in Table I.

3. Salts of the type Na_2SO_4 (*i.e.* univalent cation, bivalent anion) should allow additional swelling of gelatin chloride in concentrations from M/512 and below; in other words, Na_2SO_4 should act upon gelatin chloride as $CaCl_2$ did upon the sodium gelatinate in the previous experiments. It should form more complicated compounds symmetrical to those presumably formed by negative gelatin with Ca, expressible in the symbol $SO_4\begin{matrix}\diagup\text{gelatin}\\ \diagdown\text{gelatin}\end{matrix}$ corresponding to the symbol $Ca\begin{matrix}\diagup\text{gelatin}\\ \diagdown\text{gelatin}\end{matrix}$ discussed previously.

These predictions are fulfilled not only qualitatively but also quantitatively, as Table III shows.

In order to understand the results of this table it should be said that the preliminary perfusion with 50 cc. M/100 HCl caused a considerable swelling, due to the strong ionization of the gelatin chloride formed and the low concentration of the HCl solution used for perfusion. When such a mass containing gelatin chloride was then perfused with a neutral salt above a certain critical concentration the ionization of the gelatin chloride was diminished and hence the gelatin gave off water and the swelling was reduced. Instead of causing an additional swelling the higher concentrations of the salt, M/8 or M/16, etc., cause a shrinkage

TABLE III.

Action of Neutral Salts upon the Additional Swelling of Gelatin Chloride Produced by the Action of HCl upon Gelatin.

Additional swelling of column of 2 gm. of powdered gelatin perfused twice with 25 cc. M/100 HCl (to produce gelatin chloride) perfused subsequently four times with 25 cc. of the following solutions.

Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	H_2O
		mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
I. Univalent cation, univalent anion.	NaCl	− 3.5	−2	0	+ 4.5	12.5	21	23.5	27	30	31	31.5
	$NaNO_3$	− 6	−4	− 2.5	+ 4.5	10	17.5	25	31	33	29.5	38
	NaBr	− 8.5	−3.5	− 2.5	+ 7.0	13.5	26	42	43	56	57	54
	NaCNS	− 7	−5	− 2	0	+ 7.5	+13.5	26	25	29	27	33
	LiCl	− 6.5	−5	− 1	+ 4	10.5	18.5	24	25	29	29	27
	KBr	− 6.5	−4	+ 3.5	+ 6.5	15	20.5	35.5	33	50	41.5	37
	NH_4Br	− 8	−5	0	+ 5.5	16.5	22	24	28.5	37	36	35
	Na acetate	− 7	−5	− 2	− 2	− 4.5	0	+ 7.5	14	21	49	32
II. Univalent cation, bivalent anion.	Na_2SO_4	− 8.5	−8.5	−10	− 8	− 4.5	+ 2.5	+20	+24	40	40.5	
	Na_2 oxalate	− 7	−8	−10	− 7.5	− 9.5	− 7	+ 2.5	+22	22	26	89
	Na_2 tartrate	− 4	−6.5	− 7.5	− 8.5	− 6.0	+ 1.0	+ 3.5	+23	24	28.5	51
	Na_2 malate	−12	−8	− 9.5	− 7.5	− 6.5	+ 1.5	− 4?	+16	36	47	50
	$(NH_4)_2SO_4$	− 6	−8	− 6.5	− 5	− 3	0	+ 3	+16	23	24.5	35
III. Bivalent cation, univalent anion.	$Mg(NO_3)_2$	− 7	−7.5	− 6	− 2.5	+ 4	+14	22	34	43	44	40
	$CaBr_2$	− 9.5	−8.5	− 8	− 3.5	+ 5.5	16	28.5	37	44	67	64.5
	$Sr(NO_3)_2$	− 6.5	−7.5	− 5.0	− 3	+ 1.5	+ 8.5	16	27.5	30	35.5	39
	$BaCl_2$	− 6.5	−5.5	− 5.5	− 2.0	+ 4.0	+ 8.5	+19	31	32	34	60
	Sr acetate$_2$	− 8.5	−8	−10	−10.5	− 8.5	−10	+ 9	+20	33	35.5	49
IV. Bivalent cation, bivalent anion.	$MgSO_4$	− 7.0	−7.5	− 4	− 2	− 5	0	+ 4.5	+13	26	31	38

indicated by the negative sign in the table. This shrinkage ceases with that degree of dilution of the salt solution which no longer diminishes the original degree of electrolytic dissociation of the gelatin chloride, and with the next degree of dilution the *additional* swelling of the gelatin begins, indicated in the table by a plus sign.

We have arranged the salts in the same groups as in Table I. The first group includes the salts with univalent cation and anion. The additional swelling of gelatin chloride begins in the same dilution (with the exception of Na acetate which will be discussed later) as was the case for the sodium gelatinate in Table I; namely, at M/64. Second, it can be seen that the additional swelling begins in Group III (type $CaBr_2$) for our gelatin chloride at exactly twice the critical dilution as for Group I; namely, at M/128. This proves that regardless of the nature of the cation the additional swelling begins at exactly the same concentration of the (univalent) anion, and the value is the same for different univalent anions, for the simple reason that if a gelatin chloride is treated with a nitrate, gelatin chloride is transformed into gelatin nitrate.

Finally, the bivalent anions behave toward gelatin chloride as do the bivalent cations toward sodium gelatinate; namely, they begin to permit additional swelling at a concentration of M/512. We assumed that this behavior was due to the formation of larger complexes of gelatin symbolized in the form SO_4 gelatin$_2$ or SO_4 gelatin$_3$, which apparently do not ionize.

The acetates, Na acetate as well as Sr $(acetate)_2$, behave like the bivalent anions, for some unexplained reason possibly connected with hydrolytic dissociation.

Wherever shrinking effects of sulfates, oxalates, etc., upon the swelling of colloids actually occur, we are probably dealing with cases of protein salts of the type gelatin chloride, which are transformed into salts of the type of protein sulfate, this latter type being as incapable of swelling as salts of the type calcium gelatinate.

It might be argued that the statements of colloid chemists concerning the specific effects of anions are correct when applied to gelatin chloride. Thus the shrinking influence of the sulfates and acetates might be claimed as a confirmation of their views. Such a statement would overlook, first, the fact of the qualitatively

and quantitatively identical action of sulfates, oxalates, tartrates, malates; second, of the identical action of Cl, Br, NO_3, CNS; and third, of the identical action of Li, Na, K, NH_4, Mg, Ca, Sr, and Ba. The truth is that the influence of neutral salts upon the additional swelling of gelatin chloride is essentially of a stoichiometrical character, though for some unknown reason acetate acts abnormally.

We have assumed that gelatin chloride reacts with neutral salts only by exchanging its anion. Hardy and Pauli[8] have suggested a different reaction; namely,

$$R\begin{matrix}NH_2HCl\\COOH\end{matrix} + KCl = R\begin{matrix}NH_2HCl\\COOK\end{matrix} + HCl$$

If such a reaction takes place it is difficult to understand why $CaCl_2$ does not act in the same way upon gelatin chloride as it does upon sodium gelatinate; namely, by reducing its critical dilution (where additional swelling begins) to M/512, which is not the case. The fact that $MgSO_4$ behaves like Na_2SO_4 also suggests that only the anion of the neutral salt reacts with the gelatin chloride and that the cation does not participate in the reaction.

We, therefore, see that the limiting concentration of a neutral salt for the additional swelling of gelatin chloride could be used as a rough method for determining the molecular concentration of the salt. This is only another expression for the fact that the influence of neutral salts upon the swelling of gelatin is of a purely stoichiometrical character.

III.

When we treat gelatin with NaOH, sodium gelatinate is formed. This swells considerably, presumably on account of its high degree of electrolytic dissociation into a negative gelatin and a positive sodium ion. When we put such gelatin into a neutral salt solution that part of the swelling which is due to ionization of the protein (the "additional" swelling) should be suppressed as soon as the concentration of the salt solution reaches a certain critical value.

We assumed that when we treat gelatin with a neutral salt with bivalent cation, NaCl or K_2SO_4, sodium or potassium gelatinate is

[8] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv. 223.

formed with a high ionization constant; while the anion of the neutral salt, Cl or SO_4, does apparently not combine with the gelatin; or if it combines does not participate in the electrolytic dissociation. We have a chance to put this view to a test, since, if it is correct, gelatin treated previously with a neutral salt should behave like that treated previously with NaOH.

2 gm. of powdered gelatin were perfused twice with 25 cc. M/100 NaOH to form sodium gelatinate. The mass underwent a certain amount of swelling, the *initial* swelling, which will not be considered in the following figures. The mass was then perfused three times with 25 cc. of solution of different neutral salts, from M/8 to H_2O, to find out the limiting concentration where the different salts permit the beginning of the additional swelling. We notice that for Group I (type NaCl) this limiting concentration where the effect becomes positive is M/32; the salt with a weaker acid, Na acetate, and a weaker base, NH_4Br, forming exceptions in an opposite sense—possibly due to hydrolytic effects. The limiting concentration for salts of Group II, type Na_2SO_4, is one-half of the preceding, namely M/64; for salts of Type III ($CaCl_2$) and for Type IV it is M/256.

The relative values for these three groups are the same as for sodium gelatinate produced by the action of NaCl upon gelatin namely, NaCl: Na_2SO_4: $CaCl_2$ = 1: 2: 8.

The absolute values for the limiting concentration of these different salts for additional swelling are about but not quite twice as high for the sodium gelatinate produced by NaOH as for sodium gelatinate produced by NaCl.

Theoretical Remarks.

Our experiments were carried on with two types of gelatin salts, sodium gelatinate and gelatin chloride. The former is produced by the action of NaOH or NaCl, the latter by the action of HCl upon gelatin.

The reaction between the type sodium gelatinate and neutral salts takes place by the exchange of the cations; the anions of the neutral salts apparently not entering into the reaction with the gelatin. The reaction between gelatin chloride and neutral salts consists in an exchange between the anions, the cations apparently not participating in the reaction.

TABLE IV.

Action of Neutral Salts upon the Swelling of Sodium Gelatinate Produced by the Action of NaOH upon Gelatin.

Additional swelling of column of gelatin perfused twice with 25 cc. M/100 NaOH to produce sodium gelatinate, and then three times with 25 cc. of the following solutions.

Type of salt.	Nature of salt.	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	H_2O
		mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
I. Univalent cation, univalent anion.	KBr	− 3	− 2.5	+ 1	+ 9	+17	25	34.5	40	45	52	
	Na acetate	− 1.5	+ 1	+ 6.5	+11	16.5	21.5	29.5	39.5	41	42	39
	NH_4Br	− 4.5	− 8.0	− 2.0	+ 1.0	5.5	12.5	28.5	31.5	31.5	30.0	36
	LiCl	− 2.5	− 1.0	+ 3	10.5	12.0	51.0	55	64	59	46.6	63.5
	KCNS	− 2.5	0	+ 6.5	13	18.5	29	49	39	37.5		
	$LiNO_3$	− 2	0	+ 3	+10.5	25.5	30.5	39	38	42	43	
II. Univalent cation, bivalent anion.	Na_2 oxalate	− 2	− 1.5	+ 2.5	+ 7	+15	24	34	41	35	42	43
	K_2SO_4	− 8	− 4	− 1	+ 4	+18	19	28	34	57	50	50
	K_2 tartrate	− 5	− 9.5	+ 1.5	+ 6.5	+11.0	19.5	30.5	33.5	35.5	42.0	39.5
	Na_2 succinate	−12.0	− 5.5	− 3.5	+ 2.5	+10.5	20.5	20.5	33	35.5	35.5	35.5
III. Bivalent cation, univalent anion.	$MgCl_2$	− 7	− 7	− 6	− 6	− 4.5	0	+11	19	28	29	59
	$CaBr_2$	−12	−11	−10	−10	− 7	+ 3	+ 9.5	19	32	37	46
	$SrCl_2$	− 8	− 6.5	− 6	− 2	− 2	− 2.5	+15.5	15.5	27	35	
	$BaCl_2$	− 5	− 6	− 2.5	− 6.5	− 2	0	+13.5	16	27	30	47
IV. Bivalent cation, bivalent anion.	$MgSO_4$	−13	−10	− 8.5	− 6.5	− 4.5	+10	19	29.5	40	45	62
	$CaSO_4$					− 2.5	+ 2.5	11	23	32.5	40.5	40.5

The gelatin compounds with univalent cation or anion, *e.g.* Na gelatinate and gelatin chloride, are capable of swelling, while the gelatin compounds with bivalent anion or cation, *e.g.* Ca gelatinate and gelatin sulfate, are not. The swelling of the former two types is apparently due to ionization and we may infer that the antagonistic effect of the Ca upon sodium gelatinate and of SO_4 upon gelatin chloride is due to the formation of compounds not or less capable of ionization. Such an inference, though supported by certain facts, must, however, still be proved more directly before it can be accepted.

SUMMARY.

The new method described in previous publications for the determination of the effect of neutral salts upon the swelling of powdered gelatin has been applied to sodium gelatinate prepared by the action of M/100 NaOH and to gelatin chloride prepared by the action of M/100 HCl upon gelatin.

1. It was found that the additional swelling of gelatin chloride, produced by the action of M/100 HCl upon gelatin, is inhibited in the solutions of neutral salts of univalent metals and univalent anions (type NaCl) in concentrations above M/64 and by salts with bivalent metal and univalent anions (type $CaCl_2$) in concentrations exactly half as high, namely above M/128, no matter what the nature of cation or anion. Salts with bivalent anion (type Na_2SO_4) have a limiting concentration of a much lower order; namely, M/512.

2. It was found that the additional swelling of sodium gelatinate (produced by the action of M/100 NaOH upon gelatin) is inhibited in the solution of neutral salts of the type NaCl when the concentration is above between M/32 and M/64, and of salts of the type Na_2SO_4 when the concentration is half as high, namely above between M/64 and M/128, no matter what the nature of the cation or anion. Salts with bivalent cation, type $CaCl_2$, inhibit additional swelling in concentrations above between M/256 and M/512.

3. It was found that the additional swelling of sodium gelatinate formed by the action of high concentrations (M/8 or M/4) of NaCl upon gelatin is inhibited in the solutions of salts with univalent metal and univalent anion (type NaCl) in concentrations above M/64, and by salts with bivalent anion and univalent

cation (type Na_2SO_4) in concentrations exactly half as high; namely, above M/128. The limiting concentration of salts with bivalent cation (type $CaCl_2$) is M/512. The neutral salts, therefore, produce the same type of compounds with gelatin as the bases; namely, metal gelatinates which dissociate into a positive metal and a negative gelatin ion.

4. These facts show that the limiting concentration of neutral salts for the additional swelling is within the restrictions mentioned independent of the nature of the anion and cation of the salt and that this limiting concentration upon the swelling of gelatin could be used to calculate roughly the molecular concentration of the salt used. The influence of neutral salts upon the swelling of gelatin is therefore of a stoichiometrical character.

5. When neutral salts act upon sodium gelatinate apparently only the cation of the salt combines with the gelatin; and when neutral salts react with gelatin chloride only the anion will combine with gelatin but not the cation. When neutral salts act upon ordinary gelatin, metal gelatinates are formed which may or may not contain the anion of the salt in non- or little dissociable bondage.

6. Our experiments show that gelatin salts with univalent anion (type gelatin chloride) or with univalent cation (type sodium gelatinate) are capable of additional swelling, while salts of gelatin with bivalent anion (type gelatin sulfate) and with bivalent cation (type calcium gelatinate) are not capable of additional swelling. It has been suggested in the previous paper that this swelling of gelatin salts with univalent cation or anion is due to a high degree of electrolytic dissociation, while the lack of the power of swelling of the gelatin salts with bivalent cation or anion is due to the low degree of electrolytic dissociation of these compounds.

7. Phenomena of antagonistic salt action can be produced by the transformation of protein salts of the type sodium gelatinate which are capable of additional swelling into salts of the type calcium gelatinate not capable of additional swelling; or by the transformation of protein salts of the type of gelatin chloride capable of additional swelling into salts of the type gelatin sulfate not capable of additional swelling.

THE ESTIMATION OF NON-PROTEIN NITROGEN IN BLOOD.*

By ISIDOR GREENWALD.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

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When Folin and Denis[1] described their method for the estimation of non-protein nitrogen in blood, it became possible to make such determinations quickly, accurately, and with the use of only small amounts of blood. The method rapidly came into general use and has proved to be of great service. It has, however, not escaped modification in the interest of increased accuracy and convenience. The present author[2] pointed out that the protein precipitant should be employed in aqueous solution, so as to obtain more complete extraction of the water-soluble constituents and more complete precipitation of the fats, lipoids, etc. Trichloroacetic acid, in 2.5 per cent solution, was recommended for this purpose, the trace of protein remaining in the filtrate being removed by shaking with kaolin.

Shortly afterward, Bock[3] showed that no appreciable amount of nitrogen is split off from the blood proteins in the process of coagulation by heat. It seemed that it might be possible to free the filtrate from the coagulum of the residual protein by means of kaolin. This was found to be the case. If the filtrate from the coagulum was shaken with kaolin until the foam, which was first very voluminous, had almost completely disappeared and was then filtered, the filtrate was water-clear and gave no precipitate with $HgCl_2$, $HgI_2.2KI$, picric acid, etc., even after evaporation to a very small volume (one-tenth that of the original blood). Determinations of the total nitrogen in such filtrates gave results that agreed with those obtained by the trichloroacetic acid-kaolin method (Table I). However, upon closer study, it was found that kaolin removed some of the basic constituents of the liquid. Creatinine was found to be practically completely removed.[4] The results of some experiments upon pure materials are presented in Table II. It was evident that the use of kaolin was inadvisable.

* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 115.

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[2] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

[3] Bock, J. C., *J. Biol. Chem.*, 1916–17, xxviii, 357.

[4] A more complete discussion of the behavior of creatine and creatinine will be found in the following paper.

TABLE I.

Comparison of Values for Non-Protein Nitrogen Obtained with the Trichloroacetic Acid-Kaolin and Boiling-Kaolin Procedures.

Species.	Trichloroacetic acid-kaolin.	Boiling-kaolin.
	mg. per 100 cc.	*mg. per 100 cc.*
Human	114	112
"	30	32
"	34	38
"	43	43
"	37	36
"	60	60
"	46	46
"	33	37
"	35	36
"	38	36
"	111	98
"	43	47
"	27	30
Dog	29	29
Sheep	32	33
"	27	26
Beef	32	30

TABLE II.

Removal of Various Substances by Kaolin (20 Gm.).

Substance.	Volume of solution.	Amount taken.	Amount found.
	cc.	*mg.*	*mg.*
Creatine	470	2.66	2.65
"	500	2.83	2.81
Creatinine	500	2.35	0.10
"	500	3.57	0.10
NH_4Cl (as N)	100	1.40	1.12
NH_4Cl (as N)	1,000	54.2	50.5
Nitrogen of mixture of basic amino-acids	1,000	7.25	2.60

A number of workers[5] had found that the addition of kaolin after the use of a 2.5 per cent trichloroacetic acid was unnecessary, the trace of protein that might be present in such filtrates being

[5] Personal communications.

without appreciable effect. Upon investigation it was found that if the concentrations of the trichloroacetic acid are increased to 5 per cent, the filtrates obtained from human blood were protein-free, as far as could be determined by any of the usual tests. With other species (ox, sheep, pig, and dog), a concentration of 10 per cent is required for the same purpose. The method has been in constant use for several months and has given satisfactory results.

Since the publication of the author's previous paper on this subject, two other reagents, metaphosphoric acid and mercuric chloride, have been recommended for use in the determination of non-protein nitrogen in blood. Both of these had been tried and abandoned before the use of trichloroacetic acid had been decided upon. The former was discarded because solutions deteriorate very rapidly. One must work with freshly prepared solutions. In laboratories in which many such determinations are done, this involves little extra labor, but when such determinations are made irregularly and in comparatively small number, it is a serious hindrance. Metaphosphoric acid has been recommended by Folin and Denis,[6] who find the two filtrations formerly required in the trichloroacetic acid method objectionable. Since these have now been reduced to one, it is believed that trichloroacetic acid is the most convenient precipitant to employ in the determination of non-protein nitrogen. In all the experiments described in this paper, the nitrogen has finally been determined by distillation and titration, but, if so desired, the use of trichloroacetic acid may be combined with the direct Nesslerization procedure of Folin and Denis.

Gettler and Baker[7] have recommended the use of mercuric chloride. This had been employed by the author as a check upon his own method.[8] The amounts of nitrogen not precipitated by the two reagents were the same. Mercuric chloride was rejected as a routine reagent because it is necessary to remove or precipitate the excess of mercury before the distillation of the ammonia. Sodium sulfide and thiosulfate are generally employed for this purpose, and then the distillates contain hydrogen sulfide, from

[6] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 491.

[7] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 210.

[8] Greenwald, *J. Biol. Chem.*, 1915, xxi, 64.

which they must be freed before satisfactory titrations or colorimetric determinations can be made. Several determinations made by Gettler and Baker's method showed that from 10 to 50 per cent of the nitrogen might be left in the distillation flask, from which it could be liberated by the addition of sodium sulfide (Table III). Another, though lesser, objection to the use of hydrochloric acid and mercuric chloride is that they, in the concentrations employed by Gettler and Baker, do not completely precipitate the proteins. If potassium iodide is added to the filtrates until the precipitate of mercuric iodide is completely redissolved, it is found that the liquid is turbid and that a precipitate slowly forms. This contains from 0.7 to 2.2 mg. of nitrogen per 100 cc. of blood. If a solution of potassium-mercuric iodide is substituted for the mercuric chloride, a protein-free

TABLE III.

Results Obtained in the Determination of Non-Protein Nitrogen by the Method of Gettler and Baker, Showing the Necessity of Adding Na_2S to Precipitate the Hg.

	Mg. per 100 cc. of blood.					
Without Na_2S	21.5	31.9	23.9	30.3	9.4	18.7
Additional, after adding Na_2S	20.3	4.9	14.2	9.1	14.8	8.8

filtrate is obtained and the total nitrogen content is then the same as that obtained with the use of 5 (or 10) per cent trichloroacetic acid.

In the course of this work, a very large number of substances was employed as protein precipitants. Only a few of these require mention. Hofmeister[9] claimed that the filtrate from the heat coagulum could be freed of protein by the addition of an excess of lead carbonate or of zinc carbonate, boiling and filtering. We have not been successful with lead carbonate, but with zinc carbonate we have obtained absolutely protein-free filtrates. However, the nitrogen content of such filtrates is lower than in those obtained by the use of other precipitants. Alumina cream, whether employed as an addition to the hot coagulation mixture, or to the cold evaporated filtrate, or directly to the whole diluted

[9] Hofmeister, F., *Z. physiol. Chem.*, 1878–79, ii, 288; 1880, iv, 253.

serum, yielded filtrates that foamed freely and gave precipitates with potassium-mercuric iodide. "Dialyzed iron," employed on the evaporated filtrate from the heat coagulum, gave better results, the filtrates containing only traces of protein. The nitrogen content of such filtrates was rather low, but whether this was due to adsorption or to loss of nitrogen during the evaporation has not been established. It will be shown in the following paper that there is no appreciable adsorption of creatine or of creatinine under these conditions.

THE ESTIMATION OF CREATININE AND OF CREATINE IN THE BLOOD.*

By ISIDOR GREENWALD and GRACE McGUIRE.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

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The great interest attaching to the problems of creatinine and creatine metabolism and the failure of metabolism studies to solve them make it desirable that we should be able to study their occurrence in the tissues, including the blood. When Folin[1] described his method for this purpose, he seemed to have made it possible to determine both creatinine and creatine conveniently and accurately in small amounts of blood. The method came into general use and has yielded results of considerable interest. It calls for the treatment of the blood with four volumes of saturated picric acid solution, the addition of an excess of solid picric acid, shaking, and filtering. For the determination of preformed creatinine, 20 cc. of the filtrate are then treated with 1 cc. of 10 per cent NaOH solution and, after standing for 10 minutes, the color is compared with that of a standard solution of creatinine in saturated picric acid, similarly treated with NaOH. Total creatinine (creatine plus creatinine) is similarly determined after heating the picric acid filtrate to 120° for 20 minutes to convert the creatine into creatinine. However, there is little evidence that the substances reacting are really creatine and creatinine. The presumption is, therefore, that any method which gives lower results is more accurate, provided that it (the new method) gives good results with pure solutions and permits of quantitative recovery of the substances added to blood.

A slight modification was introduced by Myers and Fine

* A preliminary communication appeared in *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 115.

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

and Myers, Fine, and Lough,[2] who lake the blood by the addition of four volumes of water, then add dry picric acid, stirring until the protein is precipitated and a clear yellow liquid separates. The results obtained are rather higher than those obtained by the original method.

Very low values for the creatinine content of blood were reported by Gettler and Baker.[3] According to a subsequent publication by Gettler,[4] they modified the Folin method by using more alkali and also by making the color comparisons with a M 72 solution of potassium bichromate. This had previously been standardized by comparison with mixtures of known amounts of creatinine, picric acid, glucose, potassium oxalate, and sodium hydroxide solutions. It is worthy of note, however, that the amounts of glucose and of potassium oxalate employed were much greater than should be found in blood filtrates. It is generally accepted that about 0.1 per cent of oxalate is sufficient to prevent the clotting of blood.[5,6] Gettler states that 1 cc. of a saturated solution of potassium oxalate to 60 cc. of blood will prevent clotting. Since a saturated solution contains 24 per cent of the salt, this would mean a concentration of 0.4 per cent of oxalate in the blood. But in standardizing the potassium bichromate solution, Gettler used 0.1 cc. of a saturated potassium oxalate solution to the equivalent of 3 cc. of blood, a concentration of 0.8 per cent.

In making up the mixtures for standardizing the potassium bichromate solution, Gettler used 3 cc. of creatinine solution and 12 cc. of a saturated solution of picric acid, containing 100 mg. of glucose in 100 cc. The concentration of glucose, before adding the alkali, was 0.08 per cent. But, since in the determination, the blood was diluted to five times its volume, this is equivalent to a concentration of 0.4 per cent of glucose in the blood, a rather exceptional value.

Still another source of error in Gettler's work lies in the picric

[2] Myers, V. C., and Fine, M. S., *Post-Graduate*, 1915, xxx, 39. Myers, V. C., Fine, M. S., and Lough, W. C., *Arch. Int. Med.*, 1916, xvii, 570.

[3] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 214.

[4] Gettler, A. O., *J. Biol. Chem.*, 1917, xxix, 47.

[5] Hammarsten, O., Lehrb. physiol. Chem., Wiesbaden, 7th edition, 1910, 239.

[6] Mathews, A. P., Physiological Chemistry, New York, 1915, 519.

acid used. Gettler claims that this was pure. He did not use the test employed by Folin and Doisy,[7] but one of his own. The two methods were compared in this laboratory. A saturated solution of picric acid which was not particularly pure, since when mixed with 1 cc. of 10 per cent NaOH for every 20 cc. of solution and allowed to stand for 15 minutes only 9.8 mm. were required to match the color of 20 mm. of the picric acid solution alone, was examined by Gettler's method. It was found that 10 minutes after the addition of 1 cc. of 2 N NaOH to 10 cc. of this picric acid solution, the color of a layer 30 mm. high was matched by that of a layer of the standard $K_2Cr_2O_7$ solution only 6.3 mm. high. The picric acid used by Gettler and Baker, under the same conditions, required from 7.5 to 8 mm. of the bichromate solution. In view of all these sources of error it is believed that the results reported by Gettler and Baker are without significance.

The discovery by Hunter and Campbell,[8] Folin and Doisy,[7] and others that picric acid solutions, particularly when exposed to light, give rise to substances which, when treated with alkalies, give a color reaction similar to that obtained with creatinine, picric acid, and alkalies, makes it probable that the method for total creatinine is inaccurate. Fairly conclusive evidence that this is the case was furnished by Wilson and Plass.[9] They determined total creatinine in blood by removing most of the protein by coagulation by heat, following this with aluminium hydroxide, evaporating the final filtrates to small volume, and converting the creatine into creatinine by heating with 0.5 N HCl as in the original Folin method for the determination of creatine in urine. They obtained results which were considerably lower than those obtained by Folin's method. However, the objection might be raised that there may be a loss of creatine or of creatinine in the new procedure. Apparently the question was not investigated by Wilson and Plass.

More recently, Hunter and Campbell[10] have come to the conclusion that Folin's method gives results that are too high for both total and preformed creatinine. They base their conclusion upon

[7] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.
[8] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 335.
[9] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.
[10] Hunter and Campbell, *J. Biol. Chem.*, 1917, xxxii, 195.

the difference in the rate of color development in the blood filtrates and in solutions of pure creatinine in picric acid. Since this difference is even greater if the modification of Myers and Fine is employed, they believe that the higher results obtained with this modification are due to the increased participation in the reaction of some substance, not creatinine, which is liberated from the corpuscles when they are laked.

At this point, it may be well to discuss a source of error in the modification of Myers and Fine which is probably not quite so important in Folin's original procedure. In this laboratory, 10 cc. of blood are diluted with 40 cc. of water in a 100 cc. glass-stoppered Erlenmeyer flask. An excess of dry picric acid is then added and the flask is shaken thoroughly for several minutes. It may be mentioned that all of the picric acid used in this work was carefully purified and was at least as pure as that called for by Folin and Doisy. Titration of the filtrates, using methyl red as indicator, shows them to be at least 0.8 saturated. Upon a visit to another laboratory, in which the mixture of blood, water, and picric acid was merely stirred with a glass rod until all the protein was precipitated (as directed by Myers and Fine), the pale color of the filtrates attracted attention. Examination of these, both colorimetrically and titrimetrically, proved them to be only 0.1 saturated with picric acid. That a failure to achieve approximate saturation may lead to serious error is shown by the following experiment. 200 cc. of sheep blood were diluted with an equal volume of water. After the addition of an excess of dry picric acid, the mixture was shaken for several minutes and then filtered. The acidity of the filtrate was that of an 0.8 saturated solution of picric acid. From this filtrate, mixtures with water, saturated picric acid, and sodium hydroxide were prepared and the apparent creatinine content was determined with the results shown in Table I. Since the standard solution is saturated with picric acid, it is evident that the unknown should also be saturated.

Since, also, the readings obtained are inversely proportional to the creatinine content only when this is nearly the same as in the standard, it is advisable to employ a larger number of standards than were used by Folin and by Myers and Fine. In this work, standards containing 0.2, 0.4, 0.6, 1, and 2 mg. of creatinine per 100 cc. were used.

Our own experiments grew out of the observation that creatinine was almost completely removed from dilute solution after shaking with kaolin, whereas creatine was absolutely unaffected. This seemed to offer a method for the determination of creatine in blood. The blood is run into about five volumes of boiling approximately 0.03 N acetic acid, the mixture is brought to a boil, and N acetic acid added, drop by drop, until coagulation is complete. This is easily recognized by the change in the color of the foam from brown to white or colorless. The liquid is also much clearer. The mixture is filtered, the coagulum is washed with hot water, and the filtrate is cooled. After measuring the volume (or diluting it to some simple multiple of the volume of blood used),

TABLE I.

Effect of Different Concentrations of Picric Acid on the Determination of Creatinine.

	Blood filtrate, 0.8 saturated with picric acid (2 cc. = 1 cc. blood).	Water.	Saturated picric acid solution.	Final concentration of picric acid. Saturated.	Sodium hydroxide 10 per cent solution.	Creatinine found.	Creatinine per 100 cc. of blood.
	cc.	*cc.*	*cc.*		*cc.*	*mg.*	*mg.*
I	25	0	25	0.9	2.5	0.278	2.22
II	25	10	15	0.7	2.5	0.259	2.07
III	25	25	0	0.4	2.5	0.232	1.85
IV	12.5	37.5	0	0.2	2.5	0.075	1.21

it is shaken with kaolin[11] until the removal of the proteins is complete. From 5 to 10 gm. of kaolin are added at a time and thoroughly mixed with the liquid. The foam, which is at first voluminous, becomes slight and evanescent. An additional 5 or 10 gm. of kaolin are then added, also one drop of glacial acetic acid for every 100 cc. of fluid. The latter serves to agglutinate the kaolin and facilitate filtration. A volume of the water-clear, protein-free filtrate equivalent to from 10 to 40 cc. of blood is taken and evaporated, after the addition of 3 cc. of N HCl, to a volume of about 5 cc. (The smaller volumes were used with human blood; the larger with the others.) This is then kept at a tem-

[11] Eimer and Amend's acid-washed kaolin was used in most of this work. Pear's fullers' earth was also effective.

perature just below boiling for 3 hours. The low heat of an electric hot plate was found very convenient for this purpose. After cooling, a drop of 0.05 per cent solution of methyl red is added, the solution is neutralized by the cautious addition of 10 per cent NaOH solution, 15 cc. of a saturated picric acid solution are added at once, and finally 3 cc. of 10 per cent NaOH solution. After standing for 10 minutes, the solution is diluted to 100 cc. and filtered to remove a slight turbidity. The color is compared, in a Duboscq colorimeter, with that of standards similarly prepared from a dilute creatinine solution, methyl red, picric acid, and alkali. The standards are each twice the concentration of the next lower one. When only very little creatinine (0.1 mg.) was expected, as in the case of the pure solutions, the neutralized liquid was sometimes diluted to 50 cc. with saturated picric acid solution and the comparison made with solutions of creatinine in picric acid. The results summarized in Tables II, III, and IV show that the values for creatine obtained by this method must be regarded as maximal. Since they are lower than those obtained by the autoclave method, the latter must be regarded as erroneous.

An attempt was made to apply some of the methods that had been found efficacious in the removal of protein (see preceding paper) to the determination of total creatinine (creatinine and creatine). The methods employed included coagulation by heat, evaporation to about one-half the volume of the blood taken, and the removal of the residual protein with (*a*) "dialyzed iron," (*b*) the addition of trichloroacetic acid to a concentration of 10 per cent, and (*c*) direct dilution of the blood and the precipitation of the proteins by the addition of trichloroacetic acid to a concentration of 5 (or 10) per cent. All of these methods were used with animal and human bloods and found to give concordant results (Table VI). However, since the last named was also being used for the determination of non-protein nitrogen, it was the method of choice for use with human blood. 40 cc., equivalent to 4 cc. of blood, were generally used, though larger amounts were taken when available as in the work with animal bloods. As is shown in Table V, all three methods permit of quantitative recovery of creatinine or of creatine added to blood.

The creatinine content of the blood was calculated from the difference in the values obtained for total creatinine and for

creatine. Although the values obtained cannot be regarded as very accurate, they should be regarded as maximal. The essential difference in the methods employed is the use of kaolin.

TABLE II.

Removal of Creatinine from Dilute Solution by Means of Kaolin. Approximately 20 Gm. Kaolin Used. Volume of Solution, 500 Cc.

Taken.	Found.
mg.	*mg.*
1.47	<0.10
1.33	<0.10
2.35	0.30
1.30	0.10
2.35	0.10
3.57	0.18
1.30	0.06

TABLE III.

Removal, by Kaolin, of Creatinine Added to Blood.

	Creatine in 50 cc. blood.	Creatinine added.	Creatinine found.	Creatinine recovered.
	mg.	*mg.*	*mg.*	*mg.*
Beef	1.44	2.36	1.42	−0.02
"	1.44	4.71	1.55	+0.11
Pig	1.56	1.47	1.57	+0.10
Sheep	1.40	1.42	1.50	+0.10
"	1.40	2.84	1.60	+0.20

TABLE IV.

Recovery, after Treatment with Kaolin, of Creatine Added to Blood.

	Creatine in 50 cc. blood.	Added creatine (calculated as creatinine).	Expected.	Found.
	mg.	*mg.*	*mg.*	*mg.*
Sheep	2.06	2.81	4.87	4.85
"	1.45	2.81	4.26	4.35
Beef	1.67	2.81	4.48	4.58
Dog	0.80	2.65	3.45	3.39
" (25 cc.)	0.29	2.65	2.94	2.81

In addition to the substances removed by dialyzed iron or by trichloroacetic acid, this also removes basic substances. There-

fore, the difference in the values obtained is due to the removal of a basic substance which, after heating with hydrochloric acid (probably also without such heating), reacts with picric acid and

TABLE V.

Recovery, after Different Methods of Precipitating Proteins, of Creatinine and Creatine Added to Blood.

Blood contains "total creatinine."	Added creatinine as		Expected.	Found.
	Creatinine.	Creatine.		
A. Proteins precipitated by colloidal iron after heat coagulation and evaporation.				
mg.	*mg.*	*mg.*	*mg.*	*mg.*
*	0.500		0.50	0.496
*		0.566	0.566	0.564
0.48	0.50		0.98	0.89
0.48		2.83	3.31	3.15
0.81		0.69	1.50	1.46
0.40	0.37		0.77	0.76
B. Proteins precipitated by trichloroacetic acid after heat coagulation and evaporation.				
1.29	1.00		2.29	2.38
0.68	0.64		1.32	1.32
1.01	0.54		1.55	1.33
0.78	0.67		1.45	1.48
0.54	0.50		1.04	1.04
C. Proteins precipitated by trichloroacetic acid.				
†	2.00	2.83	4.83	4.66
‡	0.500		0.500	0.502
‡		0.566	0.566	0.564
2.09	0.50		2.59	2.55
2.09		0.57	2.66	2.66

* Blank using 2 cc. 0.5 M KH_2PO_4 and 2 cc. of 5 per cent dialyzed iron.

† Blank using 25 gm. of trichloroacetic acid.

‡ Blank using 12.5 gm. of trichloroacetic acid.

sodium hydroxide as does creatinine. Accordingly, the results obtained by direct precipitation of the proteins with picric acid and addition of sodium hydroxide to the filtrate must be regarded as inaccurate and, as a rule, considerably too high.

Whether or not all the color developed in the proposed procedure is due to the presence of creatinine is not so readily determined. Several attempts to isolate creatinine from blood have

TABLE VI.

Comparison of Results Obtained by the Methods of Folin and Denis and by the Proposed Methods. Results Expressed as Mg. of Creatinine per 100 Cc. of Blood.

Kind of blood.	Folin.			Proposed methods.				
				Total creatinine.				
	Creatinine.	Creatinine + creatine.	Creatine (by difference).	(a)	(b)	(c)	Creatine.	Creatinine (by difference).
Sheep, oxalated	1.13	5.78	4.65	4.36	4.29		3.28	1.08
" defibrinated	3.09	7.44	4.35	3.76	3.88	3.80	2.40	1.48
" "	3.32	7.82	5.50	3.88	4.08		2.33	1.65
Beef, oxalated	3.07	9.33	6.26	4.15	4.22	4.18	2.32	1.90
" "	2.08	10.3	8.2	4.57	4.70	4.67	3.38	1.32
" defibrinated	0.96	7.35	6.39	3.80	3.62		2.81	0.99
Calf, oxalated	2.75	8.02	5.27	3.95	3.98		3.42	0.46
" defibrinated	3.33	11.8	8.5	6.45	6.53		5.40	1.13
Pig, "	3.23	8.45	5.21	5.84	5.93	5.74	3.65	2.28
" "	3.49	8.70	5.21	5.63	5.75	5.47	3.65	2.10
Human, oxalated	2.47	4.69	2.22	2.24	2.16		1.73	0.47
" nephritic	10.3				8.81	8.13	4.1	4.7
" "	20.2	27.5	7.3	22.3	22.7	21.3	8.8	13.9
Sheep serum	1.79	4.70	2.91	3.03	3.09		1.82	1.27
" "	2.80	5.82	3.02	3.90	4.36		3.38	0.98
Beef "	2.60	6.56	3.90	5.81	5.85		4.29	1.56
" "	1.78	5.63	3.85	4.15	4.26		3.30	0.96
Pig "	4.18	6.44	2.26	4.16	4.65		3.55	1.10
" "	2.80	9.10	6.30	7.46	7.58		5.53	2.05

(a) Heat coagulation, followed by dialyzed iron.

(b) Heat coagulation, followed by trichloroacetic acid.

(c) Trichloroacetic acid directly, without previous coagulation by heat.

failed. However, there has been little evidence of the presence of other substances that would influence the results. In one experiment, 30 mg. of urea, 100 mg. of glucose, 25 cc. of Benedict and Hitchcock's uric acid standard solution[12] (containing 5 mg. of

[12] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619.

uric acid), 3 cc. of N HCl, and 400 cc. of water were evaporated and the apparent creatinine content was determined in the usual manner. 0.1 mg. was found; that is, the amount of glucose, urea, and uric acid that would ordinarily be found in 100 cc. of blood yielded only 0.1 mg. of apparent creatinine, 2 or 3 per cent of the total creatinine content of the blood. In another experiment, the same quantities were taken, with the addition of 25 gm. of trichloroacetic acid. The apparent creatinine content was 0.18 mg. A third experiment duplicated the second, except for the addition of 2.00 mg. of creatinine, and 2.83 mg. of creatine (calculated as creatinine). The amount found was 4.67 mg. instead

TABLE VII.

Rate of Color Development with Pure Creatinine and with Creatinine (?) from Blood.

Time.	Pure creatinine				Blood creatinine.			
	Apparent creatinine.	Fraction of amount in 10 min.	Apparent creatinine.	Fraction of amount in 10 min.	Apparent creatinine.	Fraction of amount in 10 min.	Apparent creatinine.	Fraction of amount in 10 min.
min.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
0.5	0.120	60	0.366	37	0.120	56	0.560	54
1	0.154	77	0.572	57	0.160	74	0.742	72
2	0.179	90	0.767	77	0.181	84	0.797	77
3	0.185	93	0.882	88	0.208	97	0.967	93
5	0.194	97	0.997	100	0.213	99	0.995	96
10	0.200	100	1.00	100	0.216	100	1.04	100
30	0.200	100	1.00	100	0.228	105	1.08	104
60	0.200	100	1.00	100	0.233	108	1.08	104

of the expected 4.83 mg. Certainly there is no appreciable error due to the presence of glucose, urea, uric acid, or any condensation products.

Following the example of Hunter and Campbell,[10] an attempt was also made to compare the rate of color development with pure creatinine and with the blood filtrate. The results obtained are summarized in Table VII and Chart 1. Into each of a series of 100 cc. volumetric flasks, there was measured a volume of solution containing 0.200 or 1.00 mg. of creatinine. To one of these there were then added 15 cc. of a saturated picric acid solution and 3.0 cc. of 10 per cent sodium hydroxide. After 10 minutes this

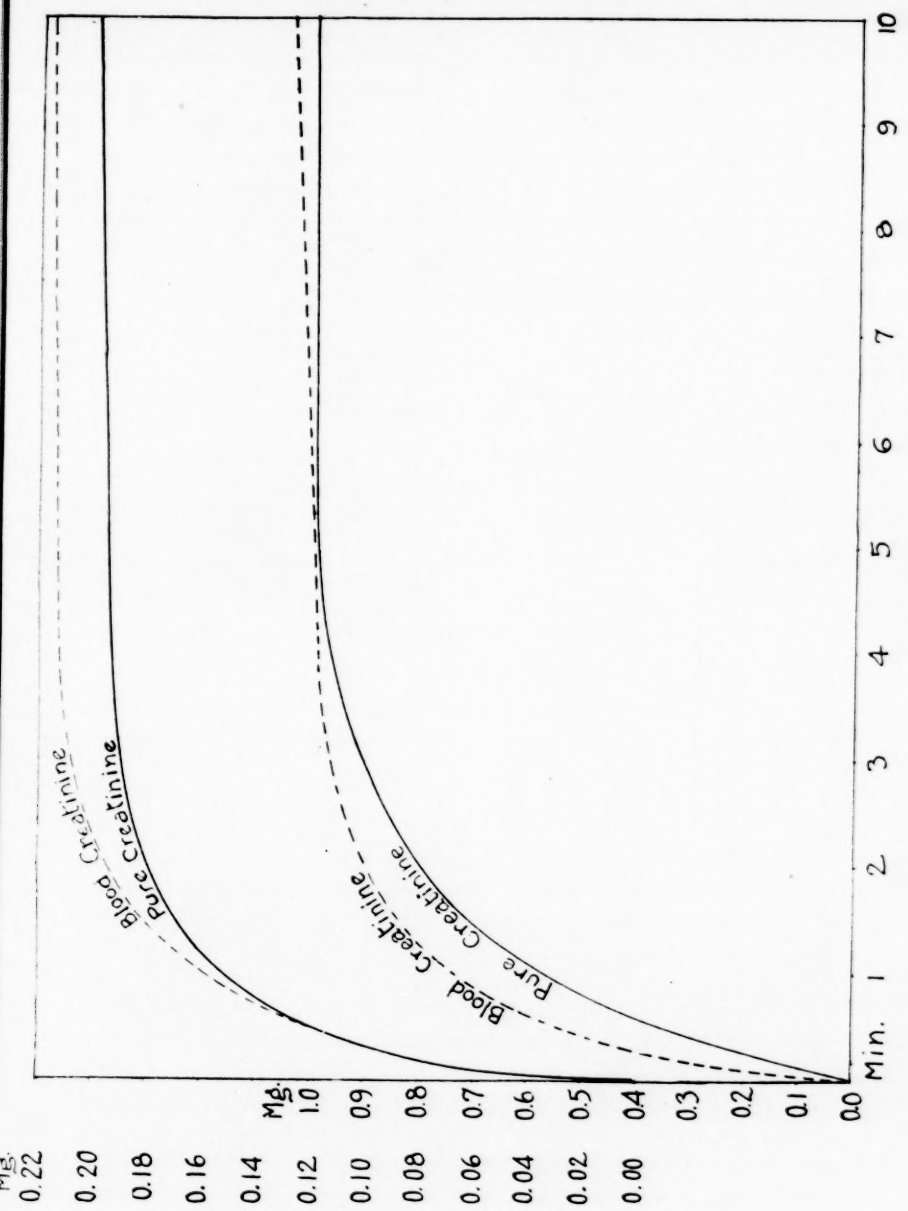
Blood Creatinine
Pure Creatinine
Blood Creatinine
Pure Creatinine
Min.
Mg.
Mg.
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
1
2
3
4
5
6
7
8
9
10

CHART 1.

was diluted to 100 cc. Similar mixtures were prepared with the same volumes of solutions, but the dilutions were made after 0.5, 1, 2, 3, 5, 30, and 60 minutes. The readings were made immediately after each dilution. The maximum color was reached in a little over 5 minutes, certainly within 10 minutes, and there was no further change for at least 1 hour. However, the velocity is not that of a monomolecular reaction, as appeared to be the case under the conditions employed by Hunter and Campbell. To study the rate of reaction with the chromogenic substance of blood, sheep blood was treated in the usual manner, using heat

TABLE VIII.

Blood from Normal Individuals. Results Expressed as Mg. of Creatinine per 100 Cc. of Blood.

No.	Folin method.			Proposed methods.		
	Creatinine.	"Total creatinine."	Creatine.	"Total creatinine."	Creatine.	Creatinine.
1	2.22	5.47	3.25	4.02	2.93	1.09
2	2.13	5.47	3.34	4.78	2.33	2.45
3	2.00	5.00	3.00	3.74	1.40	2.34
4	1.62	5.34	3.72	4.66	3.48	1.18
5	1.70	4.63	2.93		2.53	
6	2.04	5.50	3.46	3.78		
7	2.03	7.02	4.99	4.97	3.73	1.24
8	1.12			4.48	3.40	1.08
9	1.41			3.74	2.74	1.00
10	2.18			3.24	2.02	1.22
11				4.91	3.73	1.18

coagulation, "dialyzed iron," etc. After neutralization, volumes of the liquid containing approximately 0.2 or 1.0 mg. of creatinine were measured into 100 cc. flasks and the determinations made as in the case of the pure creatinine. The standards employed contained 0.200 or 1.00 mg. of creatinine. In the former case, the velocity of the reaction was the same as that obtained with pure creatinine. With the larger amount, however, there is greater divergence, the color developing more rapidly with the blood filtrate than with pure creatinine. The greater velocity may be due to the presence of another chromogenic substance, or to the presence of a catalyst (iron?). In both instances, the color changed

TABLE IX.

Blood from Hospital Patients without Nephritis. Results Expressed as Mg. of Creatinine per 100 Cc. of Blood.

No.	Diagnosis.	Non-protein nitrogen.	Uric acid.	Folin method.			Proposed methods.		
				Creatinine.	Total creatinine.	Creatine.	Total creatinine.	Creatine.	Creatinine.
1	Chronic valvular disease	37	1.1	2.47	4.69	2.22	2.20	1.73	0.47
2	Tuberculosis, pleurisy, peritonitis, passive congestion of kidney.	38	2.2	5.24	9.57	4.33	7.12	4.69	2.43
3	Tuberculosis, pleurisy, peritonitis (chest fluid)	29	1.0	1.38	3.30	1.92	3.03	1.14	1.89
4	Chronic bronchitis, emphysema, gout	45	4.8	1.96	4.03	2.07	5.46		
5	Cystitis, neurasthenia	32	3.1	6.67	11.2	4.5	4.75	3.92	0.83
6	Carcinoma of liver	34	2.3	2.44	9.66	7.22	5.32	2.43	1.89
7	Diabetes	39	6.8				3.75	2.20	1.55
8	"							2.22	
9	"	33		1.93			3.80		
10	Eosinophilia, trichinosis	35		2.55	5.85	3.30	3.44		
11	Chronic rheumatic arthritis, neurasthenia	30	3.1	2.32	5.41	3.09	3.46	1.93	1.53
12	Pyelitis	30	3.5	2.14	6.34	4.20	4.12	2.76	1.36
13	Osteomyelitis, diabetes	28	3.2	2.51	7.50	4.99	4.28	2.90	1.38
14	Chronic valvular disease, passive congestion of kidneys	33	4.6	2.17	6.90	4.73	5.87	3.47	2.40
15	Chronic valvular disease, passive congestion of kidneys	42	3.1	2.36			4.55	3.36	1.19
16	Chronic bronchitis			2.00	5.68	3.68	4.94	3.18	1.76
17	" obstructive jaundice	34	4.1	2.20	8.96	6.76	3.85	3.00	0.85
18	Dementia præcox	33	3.8	2.39			4.13	2.48	1.65
19	Chronic valvular disease, arteriosclerosis	38	4.0	1.92			6.35	5.47	0.88
20	Neurasthenia	37	3.1	1.96			5.20	2.78	2.42
21	Angina pectoris			1.37			4.21	3.43	0.78
22	Neuritis			1.52			3.94	2.26	1.68
23	Hernia			3.35			4.86	2.52	2.34
24	"			1.36			4.25	2.53	1.72
25	Chronic cardiac dilatation, passive congestion of kidneys	35	3.5	2.17			6.09	4.54	1.55
26	Polyuria, pituitary tumor (?)			2.00	6.18	4.18	5.58		
27	Arteriosclerosis	36	3.6	2.27			4.06	3.19	0.27
28	Chronic cardiac insufficiency	30	5.8	2.00			5.38	2.77	2.61

very little after 10 minutes, the slight further increase probably being due to the glucose present.

Two explanations suggest themselves for the divergence in the character of the curves obtained with blood filtrates in the experiments of Hunter and Campbell and those here reported. It may

TABLE X.

Blood from Patients with Nephritis. Results Expressed as Mg. of Creatinine per 100 Cc. of Blood.

No.	"Dye" test.	Non-protein nitrogen.	Uric acid.	Folin methods.			Proposed methods.		
				Creatinine.	Total creatinine.	Creatine.	Total creatinine.	Creatine.	Creatinine.
	per cent.								
1		38	1.8	1.72			6.24	3.55	1.69
2	51	39		3.08	9.9	6.8	6.34	4.25	2.09
3		94		1.91			4.83		
4	15	49	5.7	4.38	6.0	1.6	6.40	2.60	3.80
5	30	42	4.0	2.76	8.0	5.3	7.00	4.42	2.58
6		42		1.98	7.7	5.7	7.33		
7	13	47	3.7	3.64			9.66	2.77	6.89(?)
8		47	4.5	1.64	11.7	10.1	11.6	9.02	2.6
9		60	9.0	2.34			5.30	3.05	2.25
10	37			2.53	5.7	3.1	5.88	3.47	2.40
11	55	40		1.78	4.1	2.3	4.22		
12		67	6.8	3.75	8.0	4.2	8.47	2.57	5.90(?)
13	50	56	3.6	1.87	3.5	1.6	3.23	1.78	1.35
14	50	44	3.7	2.27	6.2	3.9	5.19	3.37	1.82
15		55	3.9	2.81	7.0	4.2	4.91	2.58	2.33
16	52			1.90	9.0	7.1	6.80	4.71	2.09
17	55	56	4.0	5.15	11.0	5.9	6.07	3.75	2.32
18	0	87	8.0	10.3			8.47	4.1	4.4
19	0	125	9.3	11.5	18.0	6.5	12.5		
20	0	126	5.1	15.9			13.2	5.9	7.3
21	0	168	6.3	16.8	24.4	7.6	19.4	8.0	11.4
22	39	58	4.5	6.54	8.3	1.8	7.75	2.5	5.3
23	0	170	6.5	20.2	27.5	7.3	22.3	8.8	13.3

be that the substance responsible for the continued development of color in the experiments of Hunter and Campbell is removed by the procedure employed in this work. It is our opinion, however, that it is more probable that the blood and picric acid react to produce a substance which, when treated with alkalies, reacts

as does the substance which is formed when a solution of picric acid is exposed to light. Another complicating factor is the uncertainty as to the actual concentration of picric acid in the filtrates employed by Hunter and Campbell (see p. 106).

Tables VIII, IX, and X present the results of a series of determinations of creatinine and creatine by Folin's method (slightly modified) and by the modifications now proposed. In many cases, the values for the non-protein nitrogen and uric acid content of the blood and for the phenolsulfonephthalein excretion are also given.[13]

From these tables we may conclude that the total creatinine content of normal human blood varies between 3 and 5 mg. per 100 cc., anything over 5 is to be regarded with suspicion, 6 is distinctly pathological, and 10 is very serious.

In normal individuals, the creatine varies between 1.4 and 3.7 mg. per 100 cc. of blood and it is not so readily increased in nephritis as is the total creatinine. A curious case is that of No. 8 in Table X. This patient showed a very high creatine by both the original Folin and the proposed methods. There is nothing in the history to suggest a reason for this exceptional occurrence.

The values for preformed creatinine, obtained as they are by difference, are rather more irregular than those for total creatinine and for creatine. They are almost always lower than those obtained by Folin's method. In the few instances, such as Nos. 7 and 12 in Table X, in which the reverse is conspicuously the case, a faulty determination of creatine or of total creatinine may be responsible.[14]

Further investigation will be required to determine the usefulness of these methods as aids to diagnosis and to the study of disease. For an investigation of normal creatine and creatinine metabolism, the methods are probably not satisfactory. Until it can be shown that the chromogenic substance is really creatinine, investigations in this field would seem to be of doubtful value.

[13] These figures were taken from the records of The Roosevelt Hospital, for permission to use which we are indebted to Dr. W. G. Lyle.

[14] The determinations upon animal blood were made in duplicate; with human blood, this was generally impossible.

We wish to acknowledge our indebtedness to the staff of The Roosevelt Hospital, and particularly to Dr. F. Hulton-Frankel and Dr. Sanford, for their assistance in obtaining specimens of blood.

STUDIES IN CALCIUM AND MAGNESIUM METABOLISM.

IV. EXPERIMENTS ON MAN.*

By MAURICE H. GIVENS.†

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

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Although one frequently meets the expressed opinion, in physiological literature, that "the calcium exchange is extraordinarily dependent upon the quality and quantity of the food ingested" (Albu and Neuberg), it is not easy to discover the precise justification for this statement. Usually the conclusion seems to be based upon assumptions rather than carefully controlled information regarding the food intake. With respect to the behavior of magnesium there is even greater uncertainty, the data regarding its elimination in cases of known intake being exceedingly scanty. The question of the possible interrelation of calcium and magnesium and even of the "normal" values for the output of these elements is still debated.

Calcium and magnesium are eliminated largely by way of the intestines, as much as 35 to 90 per cent of the lime excreted reappearing in the feces (Renvall, von Wendt). It is a generally accepted statement that the partition of excretion between kidney and gut is influenced by the intake of other salts; for example, it is alleged that an increased intake of phosphates brings out more lime through the feces. The possible rôle of the preponderance of potential acid or base in the diet in influencing the path of excretion of lime has also been discussed (Dubois and Stolte). In certain diseases, namely diabetes (Kahn and Kahn), phthisis

* A preliminary report of this work was given at the meeting of the Society of Biological Chemists, December 27–29, 1917.

† Seessel Research Fellow in Yale University.

(Senator), osteomalacia (McCrudden), and other abnormal conditions, there is little question but that a deranged metabolism of the alkali earths may occur (von Noorden).

To ascertain whether there is any "normal" range for the urinary excretion of calcium and magnesium in adult man, and what the quantitative relation of these elements to each other is, a study was made upon nine healthy laboratory workers upon whom absolute reliability could be placed.

Nelson and Williams take exception to the use of a defined and limited diet for the purpose of investigating the calcium and magnesium content of normal urine.

They state:

> "To let an individual eat what he wants and whenever he wants cannot well be done when the amount of food ingested must be measured, so most experiments necessarily represent values on more or less restricted diets, particularly regarding vegetables, and are therefore not indicative of normal values in the best sense of the word."

Obviously, however, in order to get an insight into the relation of calcium to magnesium in the urine, it is essential to have controllable conditions with respect to the intake. In the work here reported, except in regard to the amount of milk consumed, the character of the intake was not especially limited as to quality and quantity. The subjects were instructed to select from the list of articles of diet in Sherman's table those materials containing more magnesium than calcium. After a preliminary day on this diet the urine was collected for 3 days. Then the calcium-magnesium relationship in the intake was reversed by superimposing upon the diet either fresh or dried milk, calcium lactate, or magnesium citrate, and the urine was collected for another 3 day period. In the case of M. G. the urine of a 3 day period was collected on his ordinary unrestricted diet.

An examination of the list of items in Sherman's table will show that it is very easy to select a most varied diet and yet have the intake of magnesium greater than that of calcium. It is a very striking fact that were it not for the use of milk, eggs, and a few vegetables our dietaries would be decidedly poor in lime.

Nelson and Burns have come to the conclusion that, "Either calcium or magnesium may be excreted by way of the urine in the

larger amount, in the normal individual. Whichever element predominates does so constantly, or very nearly so, and seems to be independent of the character of the food ingested." This statement is not supported by the analyses here reported. In Table I are given the results of the examination of the urine of nine healthy individuals on diets having magnesium greater than calcium and then on diets having calcium greater than magnesium. That all of the individuals consumed a large amount of food is evident from the nitrogen content of the urine.

The calcium and magnesium were determined by McCrudden's method.

In only three of the nine individuals (B. C., Experiment 1, H. M., Experiment 2, and Gr.) was the urinary magnesium found in any instance to be greater than calcium, and this was only a transitory result. B. C. on a diet having magnesium greater than calcium excreted magnesium greater than calcium in the urine for the first 2 days of Experiment 1, and then on the 3rd day of the same diet the urinary calcium was greater than the magnesium. 1 month later (B.C., Experiment 2) under a similar routine this subject failed to show an excretion of magnesium greater than calcium. The response of the urinary alkali earth excretion to an increased ingestion of both elements as contained in milk is very striking. Of course the intake here was calcium greater than magnesium; and the urinary output was calcium greater than magnesium. When the increased intake was limited to one element—calcium—as in calcium lactate, the response on the part of the urinary calcium is evident, though not as striking as with the milk. The same results are found in the case of H. M. Only once did he show his urinary excretion to have magnesium greater than calcium and that on the intake of magnesium greater than calcium.

The striking effect of milk on the urinary excretion of alkali earths is further evident from the results on E. W. and A. S. When the calcium and magnesium intake was thus increased, the amount excreted through the kidneys was increased. The response to an increased intake of calcium and magnesium in a diet containing dry milk was similar to that of milk though not quite so striking. Such results were obtained with R. W. and B.

In the foregoing cases it was very easy to increase the excretion

TABLE I.

Output of Calcium and Magnesium in the Urine of Healthy Adults on Selected Diets of Naturally Occurring Foods.

Subject.	Weight.	Urine.				Diet.	
		Volume.	Total N.	Total Ca.	Total Mg.	Intake.	Supplements.
	kg.	*cc.*	*gm.*	*mg.*	*mg.*		
E. W.	47.5	1,320	10.03	120	49	Ca >Mg	Milk, 1,000 cc.
		1,350	9.45	137	57	"	" 1,000 "
		1,430	12.44	136	54	"	" 1,000 "
		1,000	9.86	54	28	Mg >Ca	
		1,035	9.45	63	37	"	
		1,450	8.50	66	50	"	
B. C. (Experiment 1).	69.0	875	13.45	98	104	"	
		975	13.82	137	144	"	
		815	11.92	95	86	"	
		995	13.58	210	126	Ca >Mg	Milk, 500 cc.
		1,135	14.95	228	162	"	" 750 "
		1,625	14.77	269	142	"	" 1,000 "
B. C. (Experiment 2).	68.5	640	10.80	100	49	Mg >Ca	
		970	14.60	137	100	"	
		1,125	14.28	144	87	"	
		1,090	11.88	171	95	Ca >Mg	0.22 gm. Ca in Ca lactate.
		1,180	12.00	192	93	"	0.42 gm. Ca in Ca lactate.
		1,140	10.26	177	74	"	0.72 gm. Ca in Ca lactate.
H. M. (Experiment 1).	75.0	755	14.70	123	108	Mg >Ca	
		1,093	14.12	103	85	"	
		690	12.79	137	108	"	
		970	15.91	189	111	Ca >Mg	Milk, 500 cc.
		730	12.29	189	109	"	" 750 "
		1,060	13.65	180	108	"	" 1,000 "
H. M. (Experiment 2).	74.4	845	12.52	111	79	Mg >Ca	
		680	11.03	122	110	"	
		705	11.24	103	110	"	
		920	13.50	119	108	Ca >Mg	0.22 gm. Ca in Ca lactate.
		960	14.00	154	118	"	0.43 gm. Ca in Ca lactate.
		1,420	16.24	150	125	"	0.72 gm. Ca in Ca lactate.

TABLE I—*Concluded.*

Subject.	Weight.	Urine.				Diet.	
		Volume.	Total N.	Total Ca.	Total Mg.	Intake.	Supplements.
	kg.	*cc.*	*gm.*	*mg.*	*mg.*		
A. S.	65.0	680	9.26	202	88	Mg >Ca	
		880	11.37	230	90	"	
		750	9.58	238	98	"	
		1,470	16.43	452	130	Ca >Mg	Milk, 1,000 cc.
		1,380	14.93	441	124	"	" 1,000 "
		1,300	13.94	369	122	"	" 1,000 "
B.	49.0	1,030	8.94	89	66	Mg >Ca	
		900	8.93	119	84	"	
		1,500	9.94	142	92	"	
		1,725	10.17	224	89	Ca >Mg	33 gm. dried milk.*
		1,060	9.44	174	75	"	66 " " "
		1,500	11.61	308	101	"	100 " " "
R. W.	75.0	1,170	9.16	91	69	Mg >Ca	
		760	9.69	115	79	"	
		1,375	9.97	125	73	"	
		1,275	12.01	154	83	Ca >Mg	33 gm. dried milk.
		1,650	13.59	192	92	"	66 " " "
		1,540	13.96	234	104	"	100 " " "
M. G.	73.0	2,250	14.96	157	118	Mg >Ca	
		2,080	13.68	127	115	"	
		2,825	14.08	155	122	"	
		2,555	13.28	222	129	Ordinary.	
		2,095	12.25	240	132	"	Milk, 120 cc.
		1,860	12.43	197	102	"	
		3,000	14.65	339	150	Ca >Mg	Milk, 500 cc.
		1,530	13.25	245	121	"	" 250 "
		2,710	13.96	292	128	"	" 500 "
Gr.	75.0	770	10.17	75	111	Mg >Ca	
		980	10.83	124	148	"	
		1,460	11.28	96	125	"	0.08 gm. Mg in 1 gm. Mg citrate.
		1,325	10.38	187	180	Ca >Mg	Milk 1,000 cc.
		1,475	10.35	169	150	"	" 1,000 "
		1,710	14.24	189	187	"	" 1,000 " +0.21 gm. Mg in 2.5 gm. Mg citrate.
S. K.	58.0	795	13.52	131	89	Mg >Ca	
		895	16.54	177	97	"	
		840	16.54	161	93	"	0.08 gm. Mg in 1 gm. Mg citrate.
		1,030	16.59	199	78	Ca >Mg	Milk, 500 cc.
		1,365	14.18	466	229	"	" 750 "

* The dried milk contained 5.66 per cent N, 1.22 per cent Ca, 0.23 per cent Mg.

of lime and alter the relation of calcium to magnesium by ingestion of either milk, skimmed or dried, or calcium lactate. The magnesium output was also slightly augmented by the increased intake of magnesium in the milk. An attempt to influence decidedly the urinary magnesium excretion was made by purposeful selection of food (M.G.) and by ingestion of a magnesium salt (Gr. and S. K.) This proved to be impossible in these cases.

M. G. never excreted more magnesium than calcium in the urine under any conditions whatsoever, even when they seemed particularly favorable for the magnesium greater than calcium relation. There was a decreased output of calcium in the urine, yet this element always preponderated. Addition of milk to the diet induced an augmented urinary output of both calcium and magnesium. Gr. responded to an intake in which magnesium was greater than calcium by excreting more magnesium than calcium in the urine, but it could not be augmented further by addition of magnesium citrate to the intake. The use of milk promptly produced a preponderance of the urinary calcium, and this was likewise unchanged by the further ingestion of magnesium citrate. S. K. failed to excrete more magnesium than calcium in the urine under any of the varied conditions of intake that were selected.

Table II gives a résumé of the figures for the urinary calcium and magnesium of normal adult human subjects reported by various investigators. Eight of the twenty writers here quoted found one or more normal individuals excreting more magnesium than calcium. Basing their conclusions primarily on the work of Neubauer, who found more magnesium than calcium excreted in the urine by normal adults, the text-books of Neumeister, Hammarsten, Neuberg, and Neubauer-Huppert contain statements that magnesium is excreted in a greater amount than calcium. Hammarsten alone has expressed the facts more adequately. He wrote: "Calcium and magnesium occur in the urine chiefly as phosphates. The quantity of earthy phosphates eliminated daily is somewhat more than a gram, and of this amount two-thirds is magnesium phosphate and one-third calcium phosphate." Such a statement does not mean that magnesium is excreted in a decidedly greater amount than calcium. A simple calculation of Neubauer's data shows that in his cases the quantity

TABLE II.

Summary of Available Data on the Output of Calcium and Magnesium in the Urine of Healthy Adults.

Investigator.	Urine.		Diet.
	Ca	Mg	
	gm.	*gm.*	
von Wendt	0.01	0.02	Sago, sugar, and butter.
Little	0.03	0.08	Vegetarian.
Sherman and collaborators	0.04	0.06	Crackers and water.
Renvall	0.05	0.03	" " "
Givens	0.05	0.03	Mixed, Mg >Ca.
Renvall	0.06	0.05	" "
Neumeister	0.06	0.12	?
Sherman and collaborators	0.07	0.08	Crackers and egg white.
von Wendt	0.07	0.05	Bread, butter, sugar, and egg white.
Nelson and Burns	0.07	0.07	Mixed.
Sherman and collaborators	0.08	0.08	Crackers, butter, and milk.
Neubauer	0.10	0.11	Mixed.
Nelson and Burns	0.10	0.19	"
Neubauer	0.10	0.14	"
Renvall	0.12	0.07	Sago, sugar, and water.
von Wendt	0.12	0.05	Bread, butter, meat, and cheese.
Nelson and Burns	0.12	0.11	Mixed.
Neubauer	0.12	0.14	"
Bertram	0.12	0.16	Meat, bread, fat, and beer.
Neumeister	0.12	0.24	?
Sherman and collaborators	0.13	0.08	Crackers, milk, butter, and water.
Boedeker	0.13	0.14	?
Sherman and collaborators	0.15	0.11	Crackers and milk.
Long and Gephart	0.15	0.12	Mixed, Ca >Mg.
Neubauer	0.15	0.16	Mixed.
Panek	0.17	0.17	?
Long and Gephart	0.18	0.12	Mixed, Ca >Mg.
Goldthwaite and collaborators	0.18	0.08	" "
Renvall	0.18	0.07	Sago, sugar, and water.
Lehmann	0.19	0.18	Bread, meat, eggs, milk (40 to 80 cc.)
"	0.19	0.14	Vegetables.
Long and Gephart	0.20	0.17	Mixed, Ca >Mg.
Gaehtgens	0.21	0.06	?
Gross	0.21	0.08	?

TABLE II—*Concluded.*

Investigator.	Urine.		Diet.
	Ca	Mg	
	gm.	*gm.*	
Sherman and collaborators	0.22	0.11	Bread and milk.
Long and Gephart	0.22	0.18	Mixed, Ca >Mg.
Bunge	0.23	0.18	Meat alone with salt and water.
"	0.24	0.08	Bread alone.
Long and Gephart	0.26	0.12	Mixed, Ca >Mg.
Panek	0.26	0.20	?
Goldthwaite and collaborators	0.26	0.18	Mixed, Ca >Mg.
Gross	0.26	0.11	?
Tenbaum	0.30	0.11	Mixed.
"	0.33	0.06	"
Labbé and Gallippe	0.34	0.19	?
Nelson and Burns	0.34	0.25	Mixed.
Beckmann	0.35	0.18	Milk, meat, cheese, and eggs.
Tenbaum	0.39	0.12	Mixed.
Givens	0.47	0.23	" Ca >Mg.
Tenbaum	0.50	0.19	"
Renvall	0.60	0.13	Cheese, ham, bread, butter, oatmeal, salt.

of the element magnesium slightly preponderated over the element calcium, the relation in general approximating 1 : 1.

In every instance cited, where the diet reported indicates an adequate intake of calcium and magnesium, with the exception of Neubauer and Little the urinary excretion of calcium is greater than that of magnesium. Bertram's diet of meat, bread, fat, beer, and sodium chloride is not a diet high in lime but high in magnesium. No explanation seems available for Neubauer's results since he only reported the diet as a "good mixed diet." In Boedeker's case the diet was not given. In the case of Little the result may be due to the fact that the subject had been a vegetarian for years. In the case of vegetarians most of the lime, it is believed, goes out through the feces (Bertram). Neubauer and Hammarsten have based their statements on the older work of Neubauer. In both the cases of von Wendt and Sherman as soon as the diet was changed to include calcium and magnesium in quantities approximating the assumed needs the urinary ex-

TABLE III.

Summary of Available Data on the Influence of Ingested Calcium and Magnesium Salts upon the Urinary Calcium and Magnesium Excretion of Healthy Adults.

Intake of Ca and Mg.	Salt added to intake.	Urine.		Investigator.
		Ca	Mg	
		gm.	*gm.*	
Usual.		0.10	0.11	Neubauer.
	$CaCO_3$	0.12	0.13	"
"		0.36	0.48	Bertram.
	$CaCO_3$	0.64	0.60	"
"		0.21		Soborow.
	$CaCO_3$	0.50		"
"		0.19		"
	$CaCO_3$	0.52		"
"		0.04–0.06		Herxheimer.
	$CaCO_3$	0.28–0.50		"
"		0.19	0.18	Lehmann.
	$CaCO_3$	0.34	0.13	"
"		0.15	0.16	Neubauer.
	$Ca_3(PO_4)_2$	0.19	0.16	"
Very low.		0.02	0.02	von Wendt.
	$CaHPO_4$	0.04	0.02	"
Low.		0.07	0.05	"
	$CaHPO_4$	0.09	0.06	"
Usual.		0.12	0.14	Neubauer.
	$CaCl_2$	0.16	0.14	"
"		0.11	0.14	"
	Ca acetate.	0.13	0.18	"
Mg >Ca.		0.11	0.10	Givens.
	Ca lactate.	0.14	0.11	"
"		0.13	0.08	"
	" "	0.18	0.09	"
Usual.		0.19	0.18	Lehmann.
	$MgCO_3$	0.20–0.29	0.23–0.28	"
Ca >Mg.		0.19	0.18	Givens.
	Mg. citrate.	0.19	0.19	"
Mg >Ca.		0.18	0.10	"
	" "	0.16	0.09	"
Usual.		0.19–0.19	0.18–0.14	Lehmann.
	$CaCO_3 + MgCO_3$	0.22–0.17	0.14–0.16	"
	$CaCO_3 + MgCO_3$	0.30	0.23	"

cretion of calcium exceeded that of magnesium. As to Nelson and Burns' results we can offer no opinion other than to express doubt as to whether the diets were adequate in their content of lime. In the case of the writer's subjects it seems that in the instances where more urinary magnesium than calcium was excreted this result was due to the diet being deficient in lime. When the lime content of the ingesta was increased and the relation of calcium to magnesium altered so that the calcium was greater than the magnesium the response in each case was immediate, with a urinary output of calcium greater than magnesium.

Table III contains the summarized published data regarding the effect of ingestion of salts upon the urinary excretion of calcium and magnesium in man. All the authors quoted in this table obtained a rise in the urinary calcium excretion after ingestion of inorganic lime salts. Our results with calcium lactate and milk, considered as a solution of calcium salts, are in accord with the foregoing. Lehmann reported an increased urinary output of magnesium after ingesting magnesium carbonate or a mixture of calcium carbonate and magnesium carbonate. With magnesium citrate no such results could be obtained in my subjects.

SUMMARY.

1. The daily urinary calcium and magnesium excretion of nine healthy adults on a diet of natural foods containing more magnesium than calcium ranged from 0.05 to 0.24 gm. Ca and from 0.03 to 0.15 gm. Mg; on a diet having more calcium than magnesium the limits were 0.12 to 0.47 gm. Ca and 0.05 to 0.23 gm. Mg. In considering the relative amounts of these elements excreted in the urine, when naturally occuring foods are eaten, it should be noted that one practically cannot increase the intake of dietary calcium without simultaneously increasing the quantity of magnesium ingested.

2. In the nine subjects here examined, in general more calcium than magnesium was excreted in the urine; or if such was not the case the usual Ca > Mg relation could be promptly brought about by ingestion of milk.

3. The urinary output of both calcium and magnesium was increased by consumption of milk. Dried skimmed milk was

practically as effective as raw milk in influencing the urinary alkali earth excretion.

4. The urinary calcium excretion was always increased after taking calcium lactate. The urinary magnesium excretion was apparently not affected by the introduction of magnesium citrate.

5. Comparable quantities of calcium salts, represented by calcium lactate, were not as efficacious as milk in producing increased urinary excretion of lime.

6. Attention is called to the variation in the relative amounts of alkali earths excreted through the kidneys by different individuals. The quantities bear no fixed relation to body weight, but presumably are influenced not only by the diet but also by the tissue reserves, particularly in the bones, of the subjects.

My thanks are due Professor Lafayette B. Mendel for his criticisms.

BIBLIOGRAPHY.

Albu, A., and Neuberg, C., Physiologie und Pathologie des Mineralstoffwechsels, Berlin, 1906.
Beckmann, W., *Centr. med. Wissensch.*, 1890, xxviii, 266.
Bertram, J., *Z. Biol.*, 1878, xiv, 335.
Boedeker, quoted by Hoppe-Seyler.
Bunge, G., Lehrb. physiol. Chem., Leipsic, 4th edition, 1898.
Dubois, M., and Stolte, K., *Jahrb. Kinderheilk.*, 1913, lxxvii, 21.
Gaehtgens, quoted by Tenbaum.
Goldthwaite, J. E., Painter, C. F., and Osgood, R. B., *Am. Med.*, 1904, vii, 547.
Gross, quoted by Neubauer-Huppert.
Hammarsten, O., Physiological Chemistry, New York, 6th edition, 1911.
Herxheimer, G., *Berl. klin. Woch.*, 1897, xxxiv, 423.
Hoppe-Seyler, F., Handb. phys. u. path. chem. Analyse, Berlin, 7th edition, 1903.
Kahn, M., and Kahn, M. H., *Arch. Int. Med.*, 1916, xviii, 212.
Labbé, H., and Gallippe, J., *Compt. rend. Soc. biol.*, 1912, lxxii, 876.
Lehmann, E., *Berl. klin. Woch.*, 1882, xix, 320.
Little, W. G., and Harris, C. E., *Biochem. J.*, 1907, ii, 230.
Long, J. H., and Gephart, F., *J. Am. Chem. Soc.*, 1912, xxxiv, 1229.
McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 187; *Arch. Int. Med.*, 1910, v, 596.
Nelson, C. F., and Burns, W. E., *J. Biol. Chem.*, 1916–17, xxviii, 237.
Nelson, C. F., and Williams, J. L., *J. Biol. Chem.*, 1916–17, xxviii, 231.
Neubauer, C., *J. prakt. Chem.*, 1856, lxvii, 65.
Neubauer-Huppert, Analyse des Harns, Wiesbaden, 1910.

Neuberg, C., Der Harn, Berlin, 1911.
Neumeister, R., Lehrb. physiol. Chem., Jena, 1897.
von Noorden, C., Lehrb. path. Stoffwechsels, Berlin, 1893.
Panek, K., *Jahresb. Fortschr. Thierchem.*, 1900, xxx, 772.
Renvall, G., *Skand. Arch. Physiol.*, 1904, xvi, 94.
Senator, H., Charité-Annalen, Berlin, 1882, vii, 397.
Sherman, H. C., Mettler, A. J., and Sinclair, J. E., *U. S. Dept. Agric., Office of Exp. Stations, Bull. 227*, 1910.
Soborow, S., *Centr. med. Wissensch.*, 1872, x, 609.
Tenbaum, E., *Z. Biol.*, 1896, xxxiii, 379.
von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211.

THE INORGANIC ELEMENTS IN NUTRITION.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

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It has long been recognized in a general way that the inorganic components of the diet play a rôle that cannot be neglected. Until recently the mineral nutrients have been largely considered *in toto* only, their content being expressed as per cent of "ash" yielded by a given food, ration, tissue, or organ. Even a casual study of the literature of nutrition impresses one with the exceedingly fragmentary and indefinite knowledge regarding the functions of the inorganic elements in the diet.

In their résumé Albu and Neuberg remarked:[1]

"So wenig wie die wissenschaftliche Medizin zurzeit den Salzen in der Ernährungsphysiologie die ihnen gebührende Bedeutung zuerkennt, würdigt sie auch die Rolle, welche sie in der Entstehung und Entwickelung von Ernährungstörungen und Stoffwechselanomalien spielen. Ohne die von Liebig, Voit und Forster in dieser Richtung gegebenen Anregungen weiter zu verfolgen, hat die chemische Stoffwechselforschung in den letzten Jahrzehnten ihre Interesse fast ausschliesslich auf die organischen Nährstoffe konzentriert. Die unorganischen Bestandteile der Nahrung und die Störungen im Umsatz derselben sind noch heute ein ziemlich brach liegendes Arbeitsfeld. Die Versuche einzelner Autoren, die Forschung auf diesem Gebiete anzuregen, sind bisher stets so fruchtlos geblieben, dass man an der Richtigkeit des Grundgedankens ernste Zweifel hegen

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A preliminary report was presented at the Minneapolis meeting of the American Society of Biological Chemists, December, 1917.

[1] Albu, A., and Neuberg, C., Physiologie und Pathologie des Mineralstoffwechsels, Berlin, 1910.

könnte, wenn man nicht wüsste, wie schwer oft Ideen Fuss fassen, welche sich nicht in der Richtung der hergebrachten Schulmeinung und der ererbten Tradition bewegen!"

This paucity of information is made evident, for example, by two recent representative text-books,[2,3] which summarize the latest conceptions in this field of physiology. Although there is almost unanimity of opinion regarding the energy needs of the body under different circumstances of age and activity; although the current estimates of the minimum amount of protein required per day seem to be defined within reasonably narrow limits; although the functions of fat and carbohydrate and the possibilities of their interchange are beginning to be understood; there is no adequate experimental basis whatever to permit tenable statements regarding either the indispensability, or the minimum requirement, of any of the inorganic constituents of the dietary with the possible exception of calcium and phosphorus. Statistics show enormous divergencies between the mineral intakes of people in different regions; but these appear to be the fortuitous results of widely unlike dietaries including water (as is the comparative dissimilarity in the fat and carbohydrate content of the diets of peoples living respectively in a tropical or frigid climate—differences enforced by the unlike character of the available food supplies) rather than the expression of unlike metabolic needs. A beginning has hardly been made in this field of investigation.

It is obvious that a growing, and bone-producing, or a milk-producing animal, requires calcium and phosphoric acid, and that at all periods of life chlorides are essential for the production of gastric juice. Conclusions like these afford an insufficient basis regarding the *specific* use of these as well as the other individual elements. Earlier observations have made it plain that strontium cannot replace calcium, and that lithium compounds are too toxic to be substituted for other alkalies. Whether the alkalies or alkali earths are to any degree interchangeable in the physiological function cannot be answered on the basis of any experimental evidence. The fetal athyrosis which results from

[2] Lusk, G., The Elements of the Science of Nutrition, Philadelphia, 3rd edition, 1917.

[3] Armsby, H. P., The Nutrition of Farm Animals, New York, 1917.

an assumed deficiency of the usual traces of iodine in the diet is an illustration of the unquestionable importance of one of the less common elements.[4]

The recent conceptions of "physiologically balanced" solutions in their relation to biological phenomena, conceptions enlarged by Loeb and others, complicate the problem considerably by forcing a consideration of the possible antagonistic actions of different ions; for in many instances physiological well-being seems to depend on a balance between stimulations and inhibitions in which the inorganic make-up of the medium may play a directive rôle. Again the modern conception of the balance of acids and bases in the body makes the interpretation of experiments affecting the inorganic metabolism of the organism difficult. Evidently it is imperative, if possible, to replace hypothesis and speculation by experimentally established facts.

The ideal method which at once suggests itself for studying the rôle of the inorganic elements individually and collectively in nutrition would consist in observing the effect of synthetic diets in which the content of every ingredient could be regulated at will both as to quantity and quality. Under such conditions the requirements of plants for the various inorganic elements were long ago demonstrated; but hitherto it has been impossible to apply similar methods to animals because mixtures of purified foodstuffs have always led to nutritive failure.

Since the demonstration[5] that comparatively small additions of yeast to otherwise suitable artificial food mixtures will render them entirely adequate for growth and maintenance, it has become possible to prepare diets from which one or another inorganic constituent can be excluded with the exception of the minute quantities introduced with the yeast and the unavoidable traces contained in the purified constituents of the food.

Accordingly we have prepared a large variety of salt mixtures in which one or more of the elements has been omitted and replaced by increments of the remaining ones so as to maintain the balance of acids and bases therein as nearly as possible. These were used to replace the *complete* mixture of inorganic salts which

[4] Smith, G. E., *J. Biol. Chem.*, 1917, xxix, 215.

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

our experiments as well as those of others have shown to be adequate for the support of white rats from infancy to maturity.

The various altered salt mixtures employed were made by dissolving the carbonates of the bases used in solutions of the required acids and then adding traces of other elements as aqueous solutions of their salts. The resultant solution of the salts thus formed was then added to lactose, evaporated until the residue was dry, pulverized, and incorporated in suitable proportions in the food.

The chemicals used in these experiments were mixed in the proportions shown in Table I.

The foods had the following general composition:

	per cent
Protein	18.0
Lactose-salt mixture	28.0–29.5
Brewer's yeast, dried	1.5– 2.0
Starch	25.5–27.5
Butter fat	18.0
Lard	7.0

The animals on these diets were given distilled water to drink.

Since in using foods from which one or more of the different inorganic ions had been intentionally omitted it was impossible to exclude traces of them occurring as impurities in the organic products used, including the yeast, a quantitative analysis was made of each ingredient of the various diets, and the total content of each element accurately determined.

K-free food (VII) contains 0.033 per cent K.* Control food (IV) contains 0.833 per cent K.*

Mg-free food (VIII) contains 0.012 per cent Mg. Control food (IV) contains 0.080 per cent Mg.

Ca-free food (IX) contains 0.008 per cent Ca. Control food (IV) contains 0.546 per cent Ca.

Na-free food (X) contains 0.035 per cent Na.* Control food (IV) contains 0.185 per cent Na.*

Cl-free food (XIB) contains 0.035 per cent Cl. Control food (IV) contains 0.529 per cent Cl.

Mg-, Na-, Cl-free food (XII) contains 0.012 per cent Mg. Control food (IV) contains 0.080 per cent Mg.

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* Na and K were not determined separately in the casein.

TABLE I.

Lactose-salt mixture	IV	VII	VIII	IX	X	XIB	XII	XIII	XV	XVI
	Control.[1]	K-free.[2]	Mg-free.[3]	Ca-free.[4]	Na-free.[5]	Cl-free.[6]	Mg-, Na-, and Cl-free.[7]	Na- and K-free.[8]	Ca-free, Mg-rich.[9]	PO_4-free.[10]
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
$CaCO_3$	13.48	13.48	16.35	0.00	14.72	13.48	13.48	13.48	0.00	12.24
$MgCO_3$	2.42	2.42	0.00	2.42	2.55	2.42	0.00	2.42	13.75	1.21
Na_2CO_3	3.42	14.26	3.42	5.80	0.00	3.42	0.00	0.00	3.42	1.71
K_2CO_3	14.13	0.00	14.13	14.13	16.66	14.13	14.13	0.00	14.13	3.53
H_3PO_4	10.32	10.32	10.32	3.72	10.32	17.50	17.50	5.16	10.32	0.00
HCl	5.34	5.34	5.34	5.34	5.34	0.00	0.00	5.34	5.34	5.34
H_2SO_4	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92
Citric acid . H_2O	11.11	11.11	11.11	11.11	11.11	11.11	6.00	0.00	11.11	9.50
Ferric citrate . $1\frac{1}{2}H_2O$	0.634	0.634	0.634	0.634	0.634	0.634	0.634	0.634	0.634	0.634
KI	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
$MnSO_4$	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079	0.0079
NaF	0.0248	0.0248	0.0248	0.0248	0.0248	0.0248	0.0248	0.0248	0.0248	0.0248
$K_2Al_2(SO_4)_2$	0.00245	0.00245	0.00245	0.00245	0.00245	0.00245	0.00245	0.00245	0.00245	0.00245
Lactose	246.0	246.0	246.0	246.0	246.0	246.0	228.0	245.0	246.0	246.0

In making all of these mixtures the chemicals were found by analysis to be pure and allowance was made for their moisture content.

[1] This mixture is based on the composition of the salts in milk.

[2] This has the K in IV replaced by an equivalent amount of Na.

[3] This has the Mg in IV replaced by an equivalent amount of Ca.

[4] This lacks the Ca in IV and three-fourths of the quantity of PO_4 required to form the $Ca_3P_2O_8$ in IV. A quantity of Na_2CO_3 equivalent to the remaining PO_4 has been added.

[5] This has the Na in IV replaced by an equivalent amount of Ca, Mg, and K added in the proportions in which they occur in IV.

[6] This has the HCl in IV replaced by an equivalent amount of H_3PO_4 calculated as a dibasic acid.

[7] This has the HCl in IV replaced by an equivalent amount of H_3PO_4 calculated as a dibasic acid. The citric acid is decreased to compensate for the removal of the Mg and Na.

[8] This has the citric acid omitted and the H_3PO_4 reduced to one-half the amount in IV to compensate for the removal of the Na and K. In making this preparation the $CaCO_3$ and $MgCO_3$ were added to the HCl and H_2SO_4, and then the H_3PO_4 was added last.

[9] This has all of the Ca of IV replaced by an equivalent amount of Mg.

[10] This has the Ca, Mg, Na, and K reduced to correspond to the H_3PO_4 removed. The citric acid is also reduced.

Mg-, Na-, Cl-free food (XII) contains 0.035 per cent Na.* Control food (IV) contains 0.185 per cent Na.*

Mg, Na-, Cl-free food (XII) contains 0.035 per cent Cl. Control food (IV) contains 0.529 per cent Cl.

Na-, K-free food (XIII) contains 0.035 per cent Na.* Control food (IV) contains 0.185 per cent Na.*

Na-, K-free food (XIII) contains 0.033 per cent K.* Control food (IV) contains 0.833 per cent K.*

Ca-free, Mg-rich food (XV) contains 0.008 per cent Ca. Control food (IV) contains 0.546 per cent Ca.

PO_4-free food (XVI) contains 0.169 per cent P in casein food. Control food (IV) contains 0.511 per cent P in casein food.

PO_4-free food (XVI) contains 0.035 per cent P in edestin food. Control food (IV) contains 0.372 per cent P in edestin food.

According to these figures none of the food mixtures were strictly Na-free, Mg-free, etc., but contained small, measured contaminations of the elements sought to be excluded, which in most were insignificant in comparison with the quantities present in our "standard" foods.

Typical results of feeding trials with these defective salt mixtures are shown in a few appended charts. In several cases the outcome was quite unexpected to us. Thus the rats on the diets low in their content of magnesium (Chart II, Rats 3773, 3774), sodium (Chart I, Rats 3854, 3848), and chlorine (Chart III, Rats 4167, 3850) respectively, grew with vigor in so far as one may judge by gains in body weight. Less than 0.04 per cent of either sodium or chlorine in the food sufficed to permit these rats to complete their growth. Indeed, one of these animals (Rat 3854 ♂, Chart I) grew to unusual size. A similar result was obtained with the diets which contained only slightly more than 0.01 per cent of magnesium (Chart II). Here, however, the relative difference between the control food and the magnesium-low food is not so great as was the case for the other elements just discussed. When all three of the elements, magnesium, sodium, and chlorine, were offered in barely more than traces, good growth was likewise possible (Chart I, Rats 4048, 4038). With only 0.03 per cent of potassium in the diet considerable growth was likewise attained (Chart I, Rats 3787, 3801), although a limit to increment of weight seemed to be imposed by the proportion afforded in these experiments. When both sodium and potassium

were simultaneously decreased in amount (Chart I, Rats 4163, 4171) growth ceased. The subsequent addition of sodium alone (Diet VII) at an early stage of growth caused only slight gain; but later, on substitution of potassium for sodium (Diet X), rapid recovery took place. After this, growth continued when sodium replaced potassium. The explanation may be that when both sodium and potassium are lacking, potassium is excreted; but when the diet contains enough sodium, potassium is tenaciously retained in the body.

In striking contrast with the foregoing experiences indicating the possibility of growth despite low levels of intake of either sodium, potassium, magnesium, or chlorine are the results of feeding diets low in calcium or phosphorus. The growth curves of Rats 3820, 3849, 3760, 3785, in Chart II, exhibit the characteristic slowing of growth which is promptly altered by the introduction of calcium in inorganic form. Our experiments also confirm those previously made by others in showing that even large additions of magnesium cannot replace calcium (see Rats 4166, 4120, Period XV, Chart II).

The lack of sufficient phosphorus in the diet is likewise promptly exhibited in a cessation or restriction of growth. From his studies of American dietaries Sherman[6] has reached the conclusion that present food habits are more likely to lead to a deficiency of phosphorus compounds than to a deficiency of protein in the diet. He regards it as not improbable that many cases of malnutrition are really due to an inadequate supply of phosphorus compounds. In our experiments where the protein furnished phosphorus, as in the case of casein, the inhibition of growth was not so pronounced as where the protein edestin, which contains no phosphorus, was furnished. With edestin foods, likewise, addition of inorganic phosphorus to the diet brought prompt responses (see Rats 4119, 4124, Chart III), thus proving that, aside from possible minute quantities, phosphorus in organic combination is not needed by the growing animal.

It has long been known that the organism can endure a considerable reduction in the customary intake of sodium chloride without any obvious untoward effects. There seems to be an

[6] Sherman, H. C., Chemistry of Food and Nutrition. New York, 1911, 265.

extraordinary capacity for adjustment to varying quantities of this salt. When an abundance is offered in the diet the elimination of chlorides through the kidneys, which are the only significant path for their excretion, is large; on a restricted chloride intake the output promptly falls (except perhaps in certain cases of pathologic "salt retention"). In starvation the output does not continue at a low level as is the case with some of the other inorganic elements and nitrogenous wastes, but sinks almost to zero. Referring to observations on man Sherman states that even where there was complete deprivation of salt during 10 to 13 days, the total loss did not exceed 10 to 15 per cent of the amount estimated as usually present in the body. Rosemann[7] has demonstrated that a diet deficient in chlorides leads at most to an insignificant reduction of the total chlorine content of the body in animals. Excretion of the element stops under such conditions; but signs of malnutrition are speedily elicited when chlorine is withdrawn from the body by actual removal of the hydrochloric acid of the gastric juice through a fistula. Until such losses are artificially enforced the gastric juice still maintains essentially its normal composition with respect to hydrochloric acid. The ability of our rats to continue in health for a time on a diet low in chlorine might have been anticipated on the basis of previous experience. It could *not*, however, have been expected that they would continue to thrive so long or attain so many times their original weight on such an extremely low chlorine intake.[8] The outcome of these experiments cannot be due to substitution of other anions for chlorine, but is attributable to a husbanding of this specific element.

What applies to chlorine is presumably true of some of the other elements like sodium, potassium, and magnesium. That these may to some degree be essential to the adjustments of neutrality regulation is indicated by the failure to grow when both sodium and potassium were practically excluded from the diet, whereas growth was nearly or quite normal when only one of these elements was missing. That these elements take part in the processes regulating the neutrality of the body fluids is to be assumed from what has been learned by experimental work *in*

[7] Rosemann, R., *Arch. ges. Physiol.*, 1911, cxlii, 208.

[8] The animal body contains about 0.2 per cent chlorine.

vitro along these lines, and also from the fact that our experiments with diets essentially free from both sodium and potassium have led to nutritive failure.

From our experiments with the so called "magnesium-free" diets, no conclusions can be drawn respecting either the persistency of its retention or the part it takes in regulating neutrality, because the amount of magnesium in the diets used was not relatively very low compared with the amount present in many ordinary foods.

That in the long run much smaller quantities of those inorganic elements which can be husbanded will be required for well-being than of those which are needed for the maintenance of neutrality and hence are continuously eliminated is manifest, wholly apart from any quantity necessary for the construction of special tissues like bone or for the production of milk.

We believe that the conventional "salt balance" experiments are less likely to permit a satisfactory elucidation of the need and rôle of the specific inorganic elements than feeding experiments, of which beginnings are here reported; for in these every factor can be better controlled. Recent studies plainly show that a deficiency of any factor essential for growth is followed by a failure of growth of the body as a whole, and not by the production of abnormal tissues due to the lack of some element. Here again the "law of minimum" reasserts itself in the part played by salts in nutrition.

The fact that the growing animal can fully supply from inorganic sources its requirements for the elements specially discussed in this paper emphasizes anew that it is unnecessary to consider the presence of calcium, phosphorus, and iron, for example, in *natural* foods to the degree that is currently believed.[9] Any shortage of an essential inorganic element can be suitably remedied under ordinary conditions by the use of its salts. For feeding farm animals, where the lack of calcium and phosphorus in their grain rations is always encountered, the demonstration that complete nutrition can be attained upon diets in which the inorganic

[9] See Sherman, H. C., and Gillett, L., The Adequacy and Economy in Some City Dietaries, New York, 1917.

ingredients are supplied in the form of their commercial salts has a significance that is just beginning to be appreciated.[10]

[10] An excellent résumé of the literature on the requirements of farm animals for mineral matter will be found in Henry, W. A., and Morrison, F. B., Feeds and Feeding, Madison, 15th edition, 1915, 62. See also numerous recent bulletins of the Ohio Agricultural Experiment Station by Forbes and collaborators.

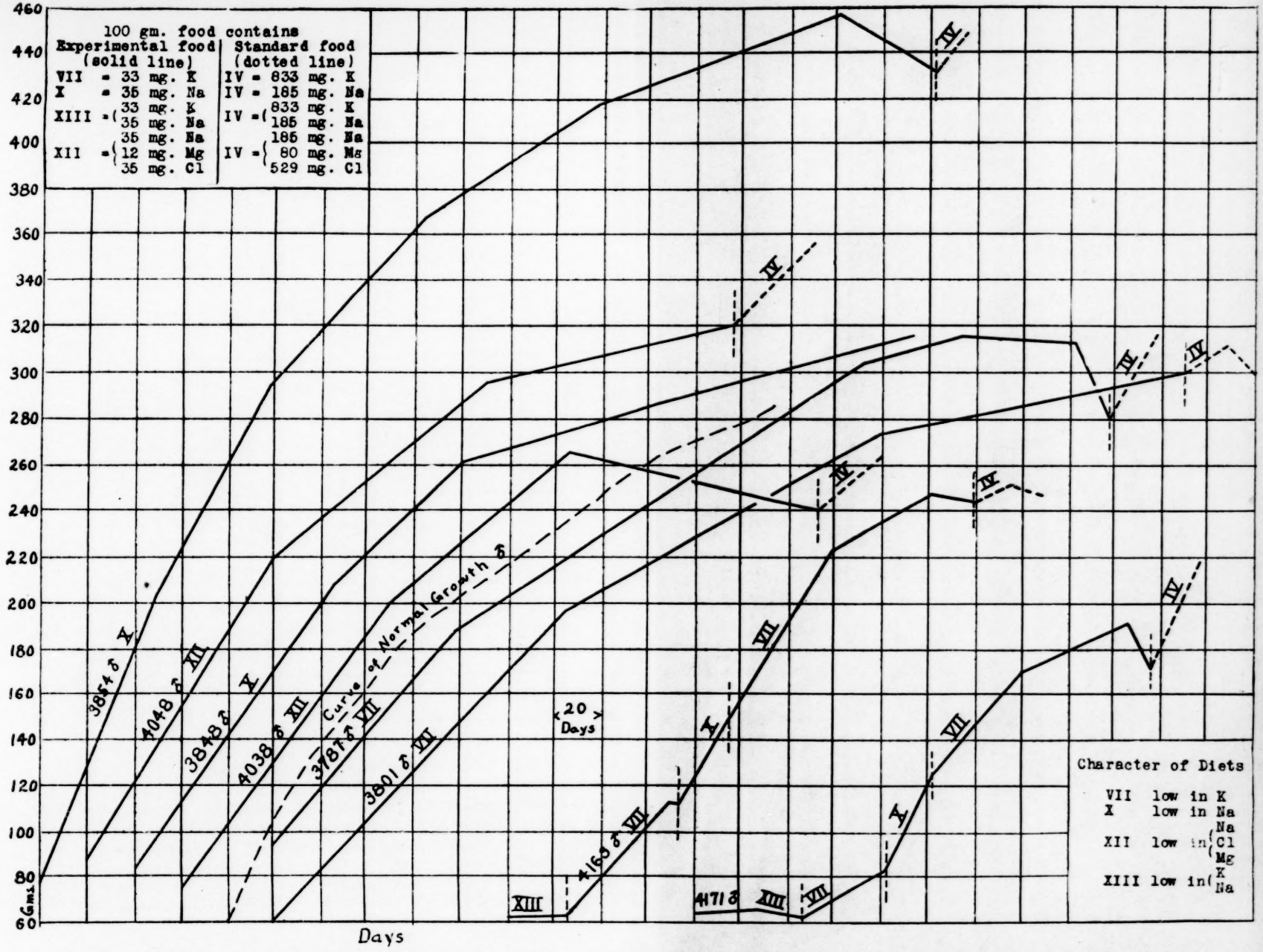
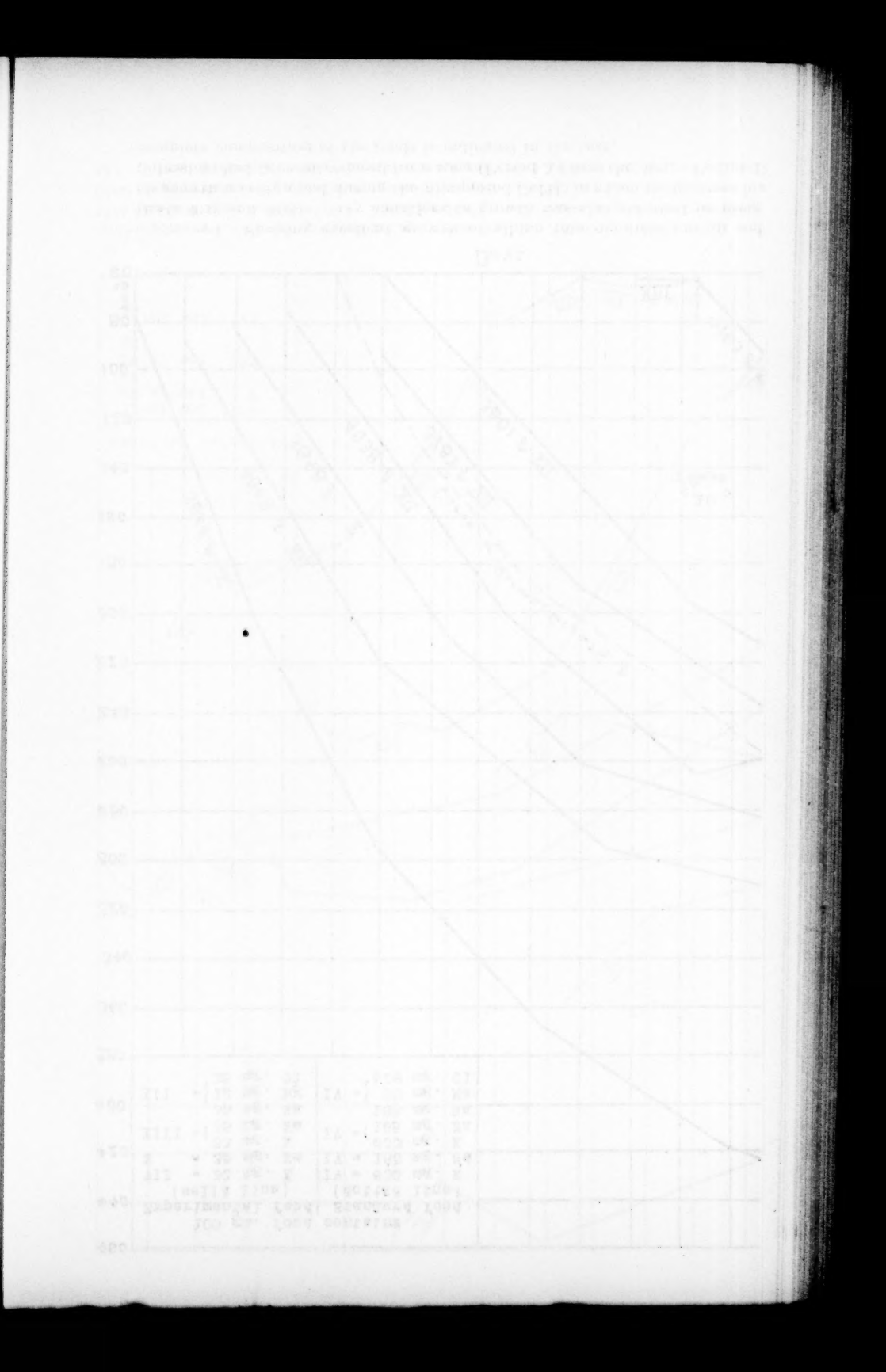

100 gm. food contains
Experimental food (solid line)
VII = 33 mg. K
X = 35 mg. Na
XIII = 33 mg. K, 35 mg. Na
XII = 35 mg. Na, 12 mg. Mg, 35 mg. Cl
Standard food (dotted line)
IV = 833 mg. K
IV = 185 mg. Na
IV = 833 mg. K, 185 mg. Na
IV = 185 mg. Na, 80 mg. Mg, 529 mg. Cl
Curve of Normal Growth ♂
3854 ♂ X
4048 ♂ XII
3848 ♂ X
4038 ♂ XII
3787 ♂ VII
3801 ♂ XII
4163 ♂ VII
4171 ♂ XIII VII
20 Days
Character of Diets
VII low in K
X low in Na
XII low in Na, Cl, Mg
XIII low in K, Na
Grms.
Days

CHART I. Showing excellent growth of albino rats on diets low in sodium (Rats 3854 and 3848), and on diets low in sodium, chlorine, and magnesium (Rats 4048 and 4038). Very considerable growth was also obtained on diets decidedly low in potassium (Rats 3787 and 3801). In the case of Rats 4163 and 4171 no growth was obtained during the first period (XIII) in which the diet was low in both sodium and potassium. Growth was not satisfactory in these animals until potassium had been introduced for a time (Period X) into the diet. Period IV at the end of the feeding trials marks the return to the "standard food." The complete composition of the foods is indicated in the text.



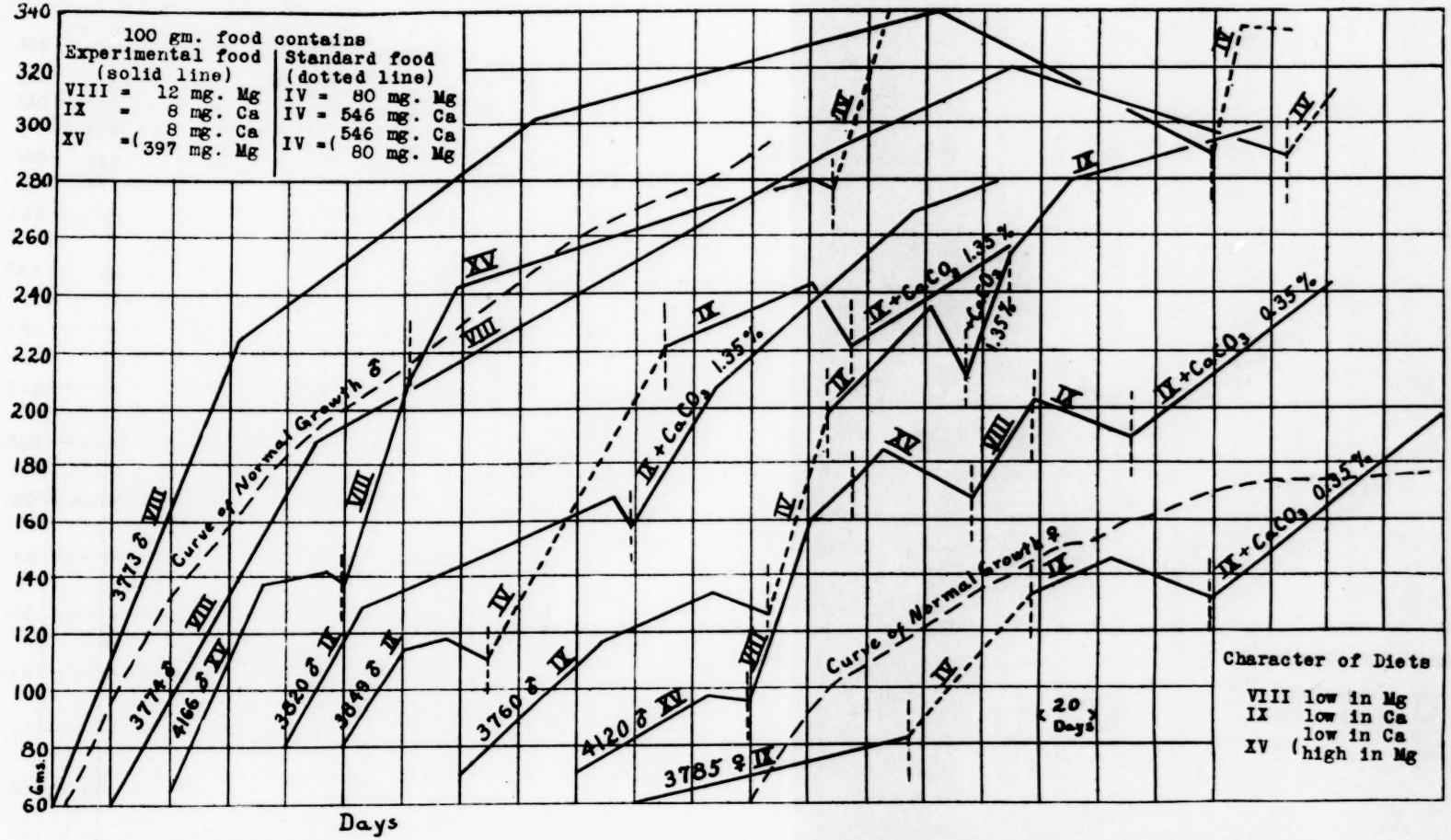

100 gm. food contains
Experimental food (solid line)
VIII = 12 mg. Mg
IX = 8 mg. Ca
XV = 8 mg. Ca, 397 mg. Mg
Standard food (dotted line)
IV = 80 mg. Mg
IV = 546 mg. Ca
IV = 546 mg. Ca, 80 mg. Mg
Curve of Normal Growth ♂
Curve of Normal Growth ♀
3773 ♂ VIII
3774 ♂ VIII XV
4166 ♂ XV
3820 ♂ IX
3849 ♂ IX
3760 ♂ IX
4120 ♂ XV
3785 ♀ IX
IX + CaCO3 1.35%
IX + CaCO3 0.35%
20 Days
Character of Diets
VIII low in Mg
IX low in Ca
XV low in Ca, high in Mg
Gms.
Days

CHART II. Showing good growth on diets comparatively low in magnesium (Rats 3773 and 3774). When the diet was low in calcium (Period IX, Rats 3820, 3849, 3760, 3785) growth invariably stopped sooner or later and usually decline ensued until calcium was reintroduced into the diet in some form. Where the "standard food" was employed the recovery was prompt and rapid (Period IV, Rats 3849, 3760, 3785). It will be noted that when restoration was brought about by addition of calcium carbonate in the periods after the decline on diets poor in calcium (Period IX, Rats 3820, 3849, 3760, 4120, 3785) the response was usually more pronounced with a higher content of calcium (1.35 per cent $CaCO_3$) than with the lower supplement (0.35 per cent $CaCO_3$). Rats 4166 and 4120 which received during Period XV a diet poor in calcium failed to thrive despite a largely augmented supply of magnesium. This is only another illustration of the now established fact that magnesium cannot replace calcium in the body. Period IV at the end of the feeding trials marks the return to the "standard food." The complete composition of the foods is indicated in the text.

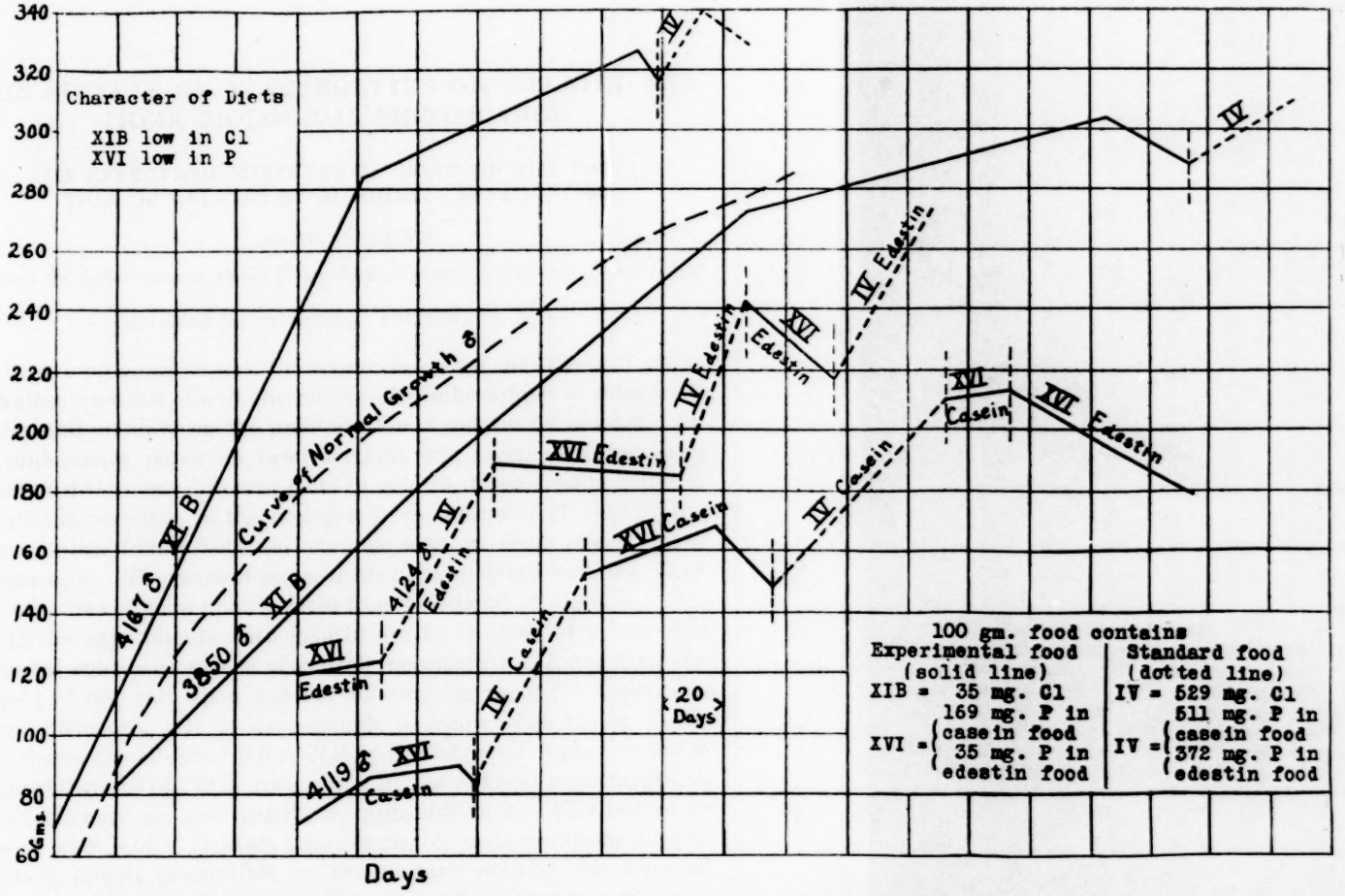


CHART III. Showing excellent growth on diets low in chlorine (Rats 4167 and 3850, Period XIB) and the failure to grow on diets poor in phosphorus (Rats 4124, 4119, Period XVI). It will be noted in the latter cases that the nutritive failure was far more rapid when the phosphorus-free protein edestin was fed than when the phospho-protein casein entered into the diet. In the Periods IV, edestin, Rat 4124, practically all of the phosphorus was supplied in inorganic form. Period IV at the end of the feeding trials marks the return to the "standard food." The complete composition of the foods is indicated in the text.

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

III. THE EFFECTS OF MIXTURES OF SALTS ON THE PRECIPITATION OF GELATIN BY ALCOHOL. ANTAGONISM.

By W. O. FENN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, February 13, 1918.)

In two previous papers, the writer described the effects of acids, alkalies, and salts,[1] and the effects of combinations of salts with acids and alkalies[2] on the precipitation of gelatin by alcohol. In a preliminary paper,[3] a brief account was given of antagonism observed between different salts in gelatin, these results being of particular interest to the biologist from the point of view of the antagonism found between various salts in their effects upon organisms. The present paper deals in more detail with the effect of mixtures of salts upon gelatin from this point of view.

If the experiments with gelatin are to be used as a basis for general conclusions, it is absolutely necessary in determining the effect of one salt upon another to combine them in all possible concentrations. It is not enough to know that 0.5 M $CaCl_2$ decreases the effect of 0.5 M NaCl; the effect of higher or lower concentrations of $CaCl_2$ upon either higher or lower concentrations of NaCl may be, and usually is, quite different. This failure to use a sufficiently representative series of concentrations of the salts is largely responsible, in the writer's opinion, for some of the conflicting results obtained in antagonism experiments.

In regard to the biological work which has been done on salt antagonism the following brief statement must suffice. Loeb[4] has

[1] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279.
[2] Fenn, *J. Biol. Chem.*, 1918, xxxiii, 439.
[3] Fenn, *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539.
[4] Loeb, J., *Proc. Nat. Acad. Sc.*, 1915, i, 473.

distinguished two kinds of antagonism: (1) that between monovalent salts (iike NaCl) and salts with bivalent or trivalent cations, and (2) that between monovalent salts and salts with bivalent or trivalent anions.

Instances of the former, or "cation antagonism," are too numerous in papers of Loeb[5] and of Osterhout[6] and of others to need mention. Cases of the latter, or "anion antagonism," are not so well known. As both cation and anion antagonism are found in gelatin, it seems desirable to review the biological evidence for the latter.

Miss Moore[7] found that Na_2SO_4 was able to antagonize the effects of NaCl on mosquito larvæ and tadpoles. NaCl alone prevents the rhythmical contraction of the lymph hearts of the frogs, but this toxicity is prevented by Na_2SO_4. Toad and frog muscles were also found to retain their vitality longer in Na_2SO_4 plus NaCl than in NaCl alone. Neilson[8] found that Na_2SO_4 and Na_3 citrate slightly antagonized the toxic effect of NH_4Cl and KCl on frog's muscle. NaCl and LiCl were not thus antagonized. Loeb and Cattell[9] observed that sodium salts hinder the poisonous effect of KCl on the heart of the embryo of *Fundulus*, the effectiveness increasing with the valency of the anion. Striking cases of anion antagonism between NaCl and Na_2SO_4, NaCl and Na_2CO_3, and Na_2SO_4 and Na_2CO_3 have been discovered by Lipman,[10] using nitrification and ammonification of soils by bacteria as criteria. Loeb,[11] on the other hand, states that mono-, bi-, and trivalent anions always failed to inhibit the toxic action of NaCl on the formation of embryos of *Fundulus*. Anion antagonism is, therefore, certainly found in biological work, but it is less pronounced than cation antagonism.

In spite of the large amount of biological work which has been done on salt antagonism, comparatively little attempt has been made, until recently, to find similar phenomena in non-living systems.

Linder and Picton (1895)[12] investigated the precipitation of arsenious sulfide in salts and mixtures of salts. They concluded that electrolytes with a positive cation (including H) were additive in their effects, but that

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[5] Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252, and literature cited there.

[6] Osterhout, W. J. V., *Bot. Gaz.*, 1906, xlii, 127; 1907, xliv, 259.

[7] Moore, A., *Am. J. Physiol.*, 1901, v, 87; 1902, vii, 315.

[8] Neilson, H., *Am. J. Physiol.*, 1902, vii, 405.

[9] Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41.

[10] Lipman, C. B., *Centr. Bakteriol.*, 1912, xxxvi, 382. Lipman, C. B., and Burgess, P. S., *ibid.*, 1914, xli, 430; 1914, xlii, 502. Lipman, *Plant World*, 1914, xvii, 295.

[11] Loeb, *Am. J. Physiol.*, 1901–02, vi, 411.

[12] Linder, S. E., and Picton, H., *J. Chem. Soc.*, 1895, lxvii, 63.

salts with monovalent cations were antagonized by salts with bivalent cations. Blake (1903)[13] confirmed these conclusions as to the antagonism between univalent and bivalent metals in a study of the effects of electrolytes on colloidal gold. Bechhold (1904)[14] and later Bender (1914)[15] studied the precipitation of colloidal mastic in mixtures of electrolytes. Pauli (1899, 1904) has studied the effects of mixtures of a few salts on the temperature of coagulation of egg white[16] and its precipitation by mixtures of salts.[17] In the latter case only did he use salts which might be expected to show antagonism, but he used comparatively few concentrations and his conclusions are not easily summarized. Hardy (1905),[18] in an investigation of the dispersing action of electrolytes on globulin, concluded that the solvent powers of two salts when acting simultaneously was the sum of their solvent powers when acting separately. The following pairs of salts he found to be additive in their dispersing action: KCl and NaCl, KCl and K_2SO_4, KCl and $MgSO_4$, NaCl and K_2SO_4, KBr and Na_2SO_4. Mines (1910)[19] presented evidence of antagonism between trivalent cations and trivalent anions. Schryver (1913)[20] discovered that the chlorides of K, Na, Mg, and Li prevented the action of $CaCl_2$ in causing solutions of sodium cholate to gel. More Ca in turn prevented the dispersing action of these salts. Clowes (1913)[21] showed that electrolytes with more strongly adsorbed cations antagonized those with more strongly adsorbed anions in their effects on oil-water systems. The anion of NaCl is regarded by him as more strongly adsorbed than the cation. Lenk (1916)[22] measured the swelling of gelatin in salts and mixtures of salts. He found less swelling in 0.2 M NaCl plus 0.2 M KCl than in 0.2 M NaCl alone. In a similar way he finds antagonism between Na_2SO_4 and NaCl, $MgCl_2$ and $MgSO_4$, NaCl and $MgSO_4$, Na_2SO_4 and $MgCl_2$, and NaCl and $CaCl_2$, but none between Ca and Hg or Ca and Mg. The writer finds the antagonism between NaCl and KCl very difficult to understand and has tried repeatedly, but in vain, to find any hint of such relationship between these salts in their effects upon the precipitation of gelatin by alcohol. Recently Loeb (1917)[23] showed that small amounts of Ca prevent the swelling of powdered gelatin which has previously been treated with NaCl.

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[13] Blake, J. C., *Am. J. Sc.*, 1903, xvi, 433.

[14] Bechhold, H., *Z. physik. Chem.*, 1904, xlviii, 385.

[15] Bender, R., *Kolloid-Z.*, 1914, xiv, 255.

[16] Pauli, W., *Arch. ges. Physiol.*, 1899, lxxviii, 333.

[17] Pauli, *Beitr. chem. Physiol. u. Path.*, 1904, v, 27.

[18] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

[19] Mines, G. R., *J. Physiol.*, 1910, xl, 327; 1911, xlii, 309.

[20] Schryver, S. B., *Proc. Roy. Soc., Series B.*, 1912–13, lxxxvi, 460.

[21] Clowes, G. H. A., *Proc. Soc. Exp. Biol. and Med.*, 1912–13, xi, 1, 4. *Kolloid-Z.*, 1914, xv, 123; *J. Physic. Chem.*, 1916, xx, 407.

[22] Lenk, E., *Biochem. Z.*, 1916, lxxiii, 15.

[23] Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

To avoid confusion it is important to define carefully our criterion of antagonism. The ideal criterion is that of Osterhout[24] who defines two electrolytes as antagonistic *if the combined effect is less than the additive effect*, the additive effect being the effect produced in a mixture when each substance acts independently of the others. When two salts act in exactly the same way so that a molecule of one may be replaced by a molecule of the other without altering the effect, the additive effect is easily determined[25] by simply adding together the concentrations of the two salts. When, however, the two salts do not act alike, *i.e.*, when the dilution curves differ as much as they often do in gelatin, the additive effect can only be approximated. It is, however, always true that the effect of two salts when they act independently, *i.e.*, the additive effect, is at least as great as the effect of the most strongly acting salt alone. It is, therefore, perfectly safe to say that *antagonism exists if the combined effect of two salts is less than the effect of the most strongly acting salt in the absence of the other* (and in the same concentration in which it exists in the combination). If the combined effect is greater than this there may still be some slight antagonism for the detection of which the additive effect must be determined. This criterion will nevertheless be used throughout this paper because of its convenience, as all the cases of antagonism observed are quite pronounced.

Description of the Method.

The details of the method of experimentation are given in the first paper of this series.[1] It consists essentially in adding 95 per cent alcohol to 5 cc. samples of gelatin dissolved in the solution of the electrolytes which it is desired to investigate until an opaque precipitate is produced. The number of cc. of alcohol required to produce a precipitate under these conditions will be called the "alcohol number." Two determinations of the alcohol number were made for each solution investigated. The fact that this method gives results which agree in general with those obtained by measurements of viscosity, temperature of gelation, and swelling proves that the alcohol number is an accurate index of the

[24] Osterhout, *Bot. Gaz.*, 1914, lviii, 178.
[25] Osterhout, *Bot. Gaz.*, 1915, lx, 228.

physical condition of the protein. This method possesses advantages over other methods in being simpler, more sensitive, and more accurate.

Statement of Results.

1. Salts (like NaCl) with monovalent anions and cations, decrease the effect on gelatin of salts with bivalent or trivalent anions or cations.

2. Salts with bivalent or trivalent cations decrease the effect on gelatin of salts with bivalent or trivalent anions.

3. Small concentrations of salts with bivalent cations (like $CaCl_2$) decrease the effect of NaCl on gelatin but small concentrations of salts with bivalent or trivalent anions (like Na_2SO_4) increase the effect of NaCl. This increase, however, is less than it would be if the NaCl were not present.

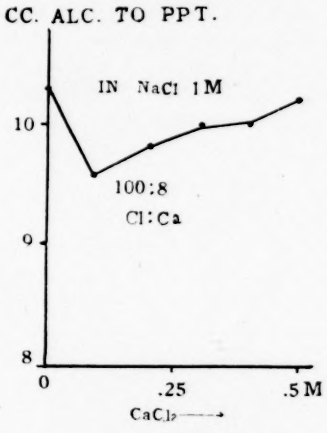

FIG. 1a.

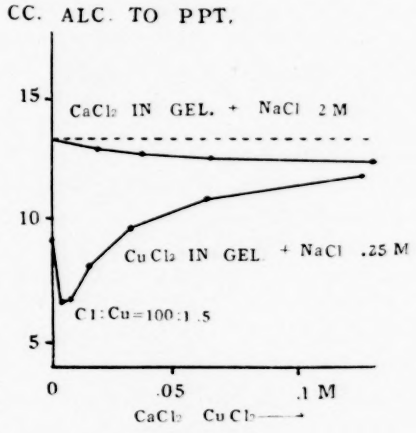

FIG. 1b.

FIG. 1a. Curve showing that small concentrations of $CaCl_2$ decrease the effect of 1 M NaCl on gelatin. This indicates antagonism. The minimum is probably an isoelectric point. At this point Cl: Ca = 100: 8.

FIG. 1b. The upper curve shows the decrease in the alcohol number caused by adding $CaCl_2$ to gelatin plus 2 M NaCl. The dotted line shows the effect of 2 M NaCl without addition of $CaCl_2$. The minimum has not yet been reached. The lower curve shows a sharp minimum caused by the addition of small concentrations of $CuCl_2$ to gelatin plus 0.25 M NaCl. At this point Na: Cu = 100: 1.5. Both curves show antagonism. (See Table I.)

TABLE I.

NaCl Antagonized by $CaCl_2$, $CuCl_2$, and $Cu(NO_3)_2$.

(1a) $CaCl_2$ in gelatin + 1 M NaCl.		(1b) $CaCl_2$ in gelatin + 2 M NaCl.		(1b) $Cu(NO_3)_2$ in gelatin + 0.132 M $NaNO_3$.		$Cu(NO_3)_2$ in gelatin.		(1b) $CuCl_2$ in gelatin + 0.25 M NaCl.*	
Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $Cu(NO_3)_2$.	Alcohol to precipitate.	Concentration of $Cu(NO_3)_2$.	Alcohol to precipitate.	Concentration of $CuCl_2$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
0.5	10.2	0.13	12.55	0.5	12.3	1.0	13.7	0.5	12.9
0.4	10.0	0.065	12.6	0.25	12.6	0.5	13.7	0.25	12.6
0.3	10.0	0.0375	12.87	0.125	13.4	0.25	14.1	0.125	11.9
0.2	9.8	0.0188	12.95	0.0625	13.9	0.125	17.6	0.0625	10.9
0.1	9.6	0.0	13.25	0.0312	13.8	0.0625	No ppt.	0.0312	9.7
0.0	10.3			0.0156	11.6	0.0312	" "	0.0156	8.1
				0.0078	8.3	0.0156	" "	0.0078	6.8
				0.0039	.3	0.0078	" "	0.0039	6.7
				0.00195	5.7	0.0039	" "	0.0	9.2
				0.0	7.6	0.00195	8.85		
						0.0	4.7		
3 per cent gelatin B at room temperature. Four determinations for each point were averaged.		2 per cent gelatin A at 30°C.		2 per cent gelatin A at 30°C. for 15 hrs.		2 per cent gelatin A at about 32°C. for 24 hrs.		2 per cent gelatin A at 22°C.° for 1 hr.	

* For the effect of $CuCl_2$ alone see the first paper[1] of this series, Table II.

In each experiment the first column shows the concentration of the variable electrolyte in the gelatin before the addition of alcohol. The second column shows the number of cc. of 95 per cent alcohol which had to be added to 5 cc. of the gelatin-electrolyte mixture in order to produce an opaque precipitate. Each figure represents the average of two determinations. The test-tubes containing the gelatin were allowed to stand at constant temperature before use in order to reach equilibrium. Gelatin A was a purer grade of commercial gelatin than B. See Fig. 1.

EXPERIMENTAL.

Salt antagonism has been studied in gelatin by the alcohol method in two ways. First, the concentration of one salt was held constant while that of the other was increased. Second, the concentrations of both salts were varied simultaneously. These two methods will be discussed in turn.

In Fig. 1a is illustrated the effect of increasing concentrations

of $CaCl_2$ (abscissæ) on the alcohol number (ordinates) of gelatin made up in molecular NaCl. At first there is a rapid drop in the curve to what is probably an isoelectric point, where the proportions of Cl to Ca are 100: 8. If the gelatin is made up in weaker NaCl, this drop in the curve disappears but antagonism is still shown by the fact that the $CaCl_2$ curve rises much less rapidly than in the absence of NaCl. This in itself is proof of the fact that NaCl decreases the effect of $CaCl_2$ on gelatin, as well as *vice versa*, but further evidence will be presented later (Figs. 8a and 8b).

Antagonism between NaCl and $CaCl_2$ is also shown in the upper curve in Fig. 1b where the addition of $CaCl_2$ to gelatin plus 2 M NaCl causes a decrease in the alcohol number. The dotted line shows the effect of 2 M NaCl without addition of $CaCl_2$. The minimum of the curve has not been reached as there are not yet as many as eight atoms of Ca to 100 of Cl, this being approximately the necessary proportion for the isoelectric point.

In a previous paper, $CuCl_2$ was shown to be much more effective than $CaCl_2$ in preventing the precipitation of gelatin by alcohol. It is to be expected, therefore, that less Cu than Ca would be required to bring the gelatin to the isoelectric point. This expectation is verified in the lower curve in Fig. 1b where the effect of increasing concentrations of $CuCl_2$ on the alcohol number of gelatin plus 0.25 M NaCl is shown. The curve is seen to pass through a sharp minimum at a point where the proportions of Cl to Cu are 100: 1.5. After the minimum, the curve rises again but not so rapidly or so high as if the $CuCl_2$ were present without NaCl. In pure $CuCl_2$ no precipitate at all can be obtained with alcohol, between the concentrations of 0.002 and 0.125 M. In Table I are also given figures for the effect of $Cu(NO_3)_2$ on the alcohol number of gelatin plus 0.132 M $NaNO_3$ and for $Cu(NO_3)_2$ alone. These figures closely resemble those for $CuCl_2$. The proportions of NO_3 to Cu at the minimum are in this case 100: 2.7.

In Fig. 2 are three curves showing the effects of $AlCl_3$ on gelatin dissolved in water, in 1.5 M $CaCl_2$, and in 2 M NaCl respectively. Above the concentration of $AlCl_3$ where the broken line begins in the water curve, no precipitation of the gelatin by alcohol was possible. In the presence of NaCl or $CaCl_2$ precipitation occurs.

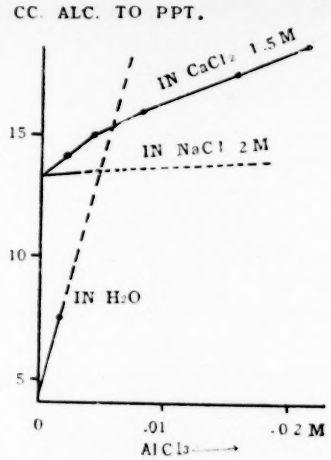

FIG. 2. Curves showing that increasing concentrations of $AlCl_3$ entirely prevent the precipitation of gelatin by alcohol (broken line). 1.5 M $CaCl_2$ and 2 M NaCl make precipitation possible by their antagonistic action on $AlCl_3$. The dotted line indicates that gelatin is precipitated by $AlCl_3$ + NaCl in these concentrations without alcohol. (See Table II.)

TABLE II.

2 M NaCl and 1.5 M $CaCl_2$ Decreasing the Effect of $AlCl_3$.

$AlCl_3$ in gelatin.		$AlCl_3$ in gelatin + 2M NaCl (antagonistic).		$AlCl_3$ in gelatin + 1.5 M $CaCl_2$ (antagonistic).	
Concentration of $AlCl_3$.	Alcohol to precipitate.	Concentration of $AlCl_3$.	Alcohol to precipitate.	Concentration of $AlCl_3$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.
0.0022	No ppt.	0.0032	Ppt. without alcohol.	0.0625	No ppt
0.0011	6.85	0.0016	14.0	0.0312	22.5
0.00056	5.55	0.0008	13.7	0.0156	17.5
0.00028	4.95	0.0004	13.5	0.0078	17.0
0.00014	4.65	0.0002	13.45	0.0039	14.9
0.0	4.4	0.0	13.25	0.00195	13.9
				0.00097	13.6
				0.0	13.2
2 per cent gelatin A at 30°C. for 15 hrs.		2 per cent gelatin A at 30°C. for 19 hrs.		2 per cent gelatin A. Temperature varied previous to titration because regulator broke.	

For other details see Table I. See Fig. 2.

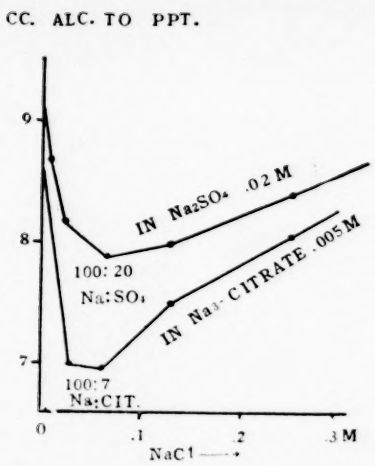

FIG. 3. Curves showing that small concentrations of NaCl decrease (*i.e.* antagonize) the effect of Na_2SO_4 and Na_3 citrate on the alcohol number of gelatin. The proportions of Na to SO_4 and Na to citrate at the minima are 100: 20 and 100: 7 respectively. (See Table III.)

TABLE III.

NaCl Decreasing the Effect of Na_2SO_4 and Na_3 Citrate.

NaCl in gelatin + 0.02 M Na_2SO_4.	
Concentration of NaCl.	Alcohol to precipitate.
M	cc.
0.5	9.5
0.25	8.4
0.125	8.0
0.0625	7.9
0.0312	8.2
0.0156	8.3
0.0078	8.8
0.0039	8.9
0.00195	9.0
0.00097	9.1
0.0	9.2

3 per cent gelatin C at 26°C. for 20 hrs.

NaCl and KCl in gelatin + 0.005 M Na_3 citrate.	
Concentration of NaCl.	Alcohol to precipitate.
M	cc.
0.5	9.25
0.25	8.05
0.125	7.45
0.0625	6.95
0.0312	7.0
Concentration of KCl.	
0.5	9.2
0.25	8.3
0.125	7.35
0.0625	6.85
0.0312	7.05
0.0	8.6

3 per cent gelatin B at 26°C. for 24 hrs.

Other details as in Table I. See Fig. 3.

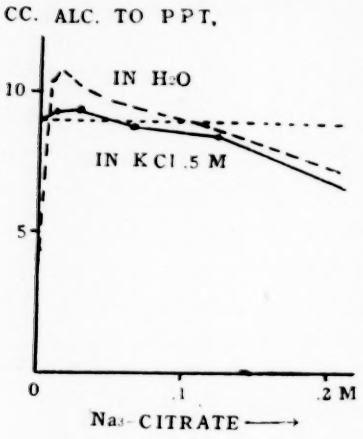

FIG. 4.

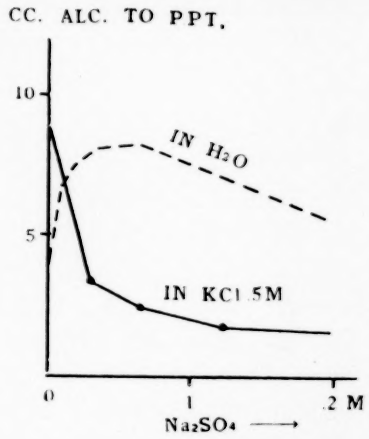

FIG. 5.

FIG. 4. Curves showing that in the presence of 0.5 M KCl, the maximum of the Na_3 citrate curve (broken line) is lowered. This shows antagonism. The curve shows also that small concentrations of Na_3 citrate cannot cause a decrease of the effect of KCl as $CaCl_2$ can. (See Table IV.)

FIG. 5. This curve shows that in the presence of 0.5 M KCl the maximum of the Na_2SO_4 curve has disappeared. The decrease which is shown here is the same as the decrease in the alcohol number in high concentrations of pure Na_2SO_4. (See Table IV.)

TABLE IV.

Na_2SO_4 and Na_3 Citrate in Gelatin + 0.5 M KCl.

Concentration of Na_2SO_4.	Alcohol to precipitate.	Concentration of Na_3 citrate.	Alcohol to precipitate.
M	cc.	M	cc.
0.25	1.6	0.25	5.9
0.125	1.8	0.125	8.4
0.0625	2.3	0.0625	8.8
0.0312	3.2	0.0312	9.2
0.0156	5.6	0.0156	9.3
0.0078	8.2	0.0078	9.2
		0.0	9.0

3 per cent gelatin B at 26°C. for 30 hrs.

Other details as in Table I. See Figs. 4 and 5.

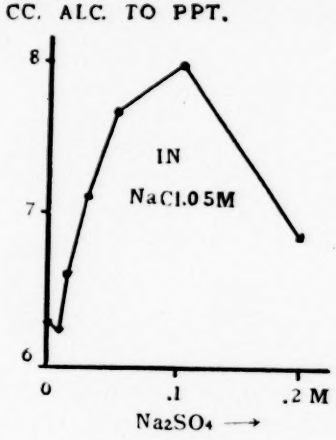

FIG. 6.

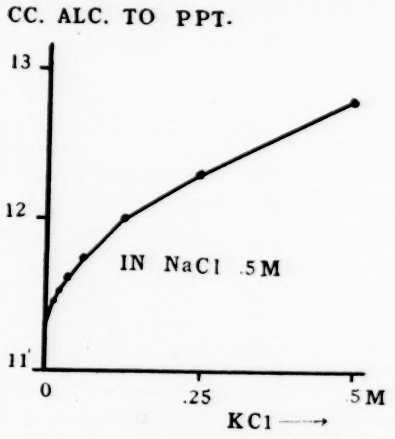

FIG. 7.

FIG. 6. This curve shows that small concentrations of Na_2SO_4 (unlike $CaCl_2$) increase the alcohol number of gelatin plus 0.05 M NaCl. They would increase the alcohol number of pure gelatin still more. The small initial decrease is due probably to the acidity of the gelatin and is not significant. (See Table V.)

FIG. 7. Curve showing that no decrease in the alcohol number is obtained by adding KCl to gelatin plus 0.5 M NaCl. KCl and NaCl are therefore not antagonistic but interchangeable in their effects. (See Table V.)

TABLE V.

Na_2SO_4 and KCl in Gelatin + NaCl.

Na_2SO_4 in gelatin + 0.05 M NaCl.		KCl in gelatin + 0.5 M NaCl (additive).	
Concentration of Na_2SO_4.	Alcohol to precipitate.	Concentration of KCl.	Alcohol to precipitate.
M	cc.	M	cc.
0.189	6.8	0.5	12.8
0.095	7.95	0.25	12.3
0.048	7.65	0.125	12.0
0.024	7.1	0.0625	11.7
0.012	6.6	0.0312	11.6
0.006	6.2	0.0156	11.5
0.003	6.25	0.0078	11.4
0.0	6.30	0.0	11.3
2 per cent gelatin A at 30°C. Control is average of four determinations.		2 per cent gelatin A at 30°C.	

Other details as in Table I. See Figs. 6 and 7.

The effect of NaCl is greater than that of $CaCl_2$ in antagonizing the $AlCl_3$ and making precipitation by alcohol possible. The action of the two is not the same, however, because small amounts of $CaCl_2$ would be expected to increase the effect of small amounts of $AlCl_3$ on gelatin, if $AlCl_3$ behaves like HCl in this respect as it does in others. Furthermore, $AlCl_3$, when combined with NaCl in the proper proportions is a strong precipitant for gelatinl but not when combined with $CaCl_2$. This indicates that NaC, can make gelatin plus $AlCl_3$ isoelectric, but $CaCl_2$ cannot. NaCl, therefore, decreases the effect of $AlCl_3$ on gelatin. $AlCl_3$, however, slightly increases the effect of 2 M NaCl on gelatin as seen by the slight rise of the NaCl curve. With weaker NaCl, the rise would be correspondingly greater, but it is always less than would be expected if no NaCl were present.

It has already been shown that NaCl in gelatin made up in acid,[2] alkali,[2] $AlCl_3$ (Fig. 2), or $CaCl_2$ (Fig. 1, see also Fig. 8) can decrease the alcohol number. Fig. 3 shows that this is also true for gelatin dissolved in 0.02 M Na_2SO_4 and 0.005 M Na_3 citrate. As the concentration of NaCl is increased, there is a rapid decrease in the alcohol number until a minimum is reached. This, again, is probably an isoelectric point. The proportions of Na to SO_4 or citrate at this point are 100: 20 and 100: 7 respectively. These proportions are about what would be expected. The citrate ion, being trivalent and giving an alkaline solution on hydrolysis, requires more Na to "neutralize" it than the SO_4 ion.

Na_2SO_4 and Na_3 citrate are, therefore, undoubtedly antagonized by NaCl, but unlike $CaCl_2$ they are unable to cause a decrease in the alcohol number when added in small amounts to gelatin plus NaCl. This is shown for Na_3 citrate and KCl in Fig. 4. The horizontal dotted line (showing the effect of pure 0.5 M KCl) is drawn to show that the first effect of the Na_3 citrate is to cause an increase in the alcohol number above that caused by KCl alone. This increase, however, is not so great as would be expected for pure Na_3 citrate without NaCl. (The curve for Na_3 citrate alone is shown in broken line.) The fact that the presence of KCl lowers the maximum of pure Na_3 citrate is proof of antagonism.

The greater the concentration of KCl in the gelatin, the less the initial increase in the alcohol number and the sharper the sub-

sequent decrease. Finally, a point is reached where the initial increase disappears altogether and only the subsequent decrease remains. With Na_2SO_4, which has less effect when acting alone than Na_3 citrate, less KCl is necessary to reach this point. Fig. 5 shows that 0.5 M KCl is sufficient. The decrease in the alcohol number, shown here, when Na_2SO_4 is added to gelatin plus KCl, is due to the same factor that causes the decrease in the alcohol number in high concentrations of Na_2SO_4 alone. It is not comparable to the decrease caused by $CaCl_2$ in Fig. 1.

On the other hand, if the concentration of the KCl in the gelatin in Fig. 4 is decreased, the initial increase becomes greater. It is always, however, less than would be expected if there were no antagonism. Such a case is shown for Na_2SO_4 and NaCl (which acts like KCl) in Fig. 6. The very small initial decrease in this experiment is probably due to the acidity of the gelatin, as a similar drop is obtained with small amounts of NaOH in gelatin. It is scarcely greater than experimental error and is certainly not comparable to the decrease caused by the addition of $CaCl_2$ to gelatin plus NaCl as in Fig. 1.

NaCl is therefore antagonistic to $CaCl_2$ and to Na_2SO_4, but small concentrations of Na_2SO_4 cannot decrease the effect of NaCl on gelatin as $CaCl_2$ can. This difference between $CaCl_2$ and Na_2SO_4 is found also in the fact (not yet published) that Na_2SO_4 tends to hinder the precipitation of gelatin by NaCl, while $CaCl_2$ assists it.

In view of what was said at the beginning of this paper on the necessity of using a sufficient variety of concentrations in determining the effect of one salt upon another, it is worth while to draw the attention of the reader to the conflicting conclusions which might be arrived at as to the relations between NaCl and Na_2SO_4, for example, if only a single concentration of each salt had been used. This is shown by the number of maxima and minima which appear in the curves plotted in Figs. 3, 4, 5, and 6. This is only a single example of what will be found true throughout these experiments.

If two monovalent salts, such as KCl and NaCl, or $NaNO_3$ and KCl, are used, the concentration of one being held constant while that of the other increases, there is merely an increase in the alcohol number which gradually reaches a maximum. Such salts are, therefore, not antagonistic to each other. Such an

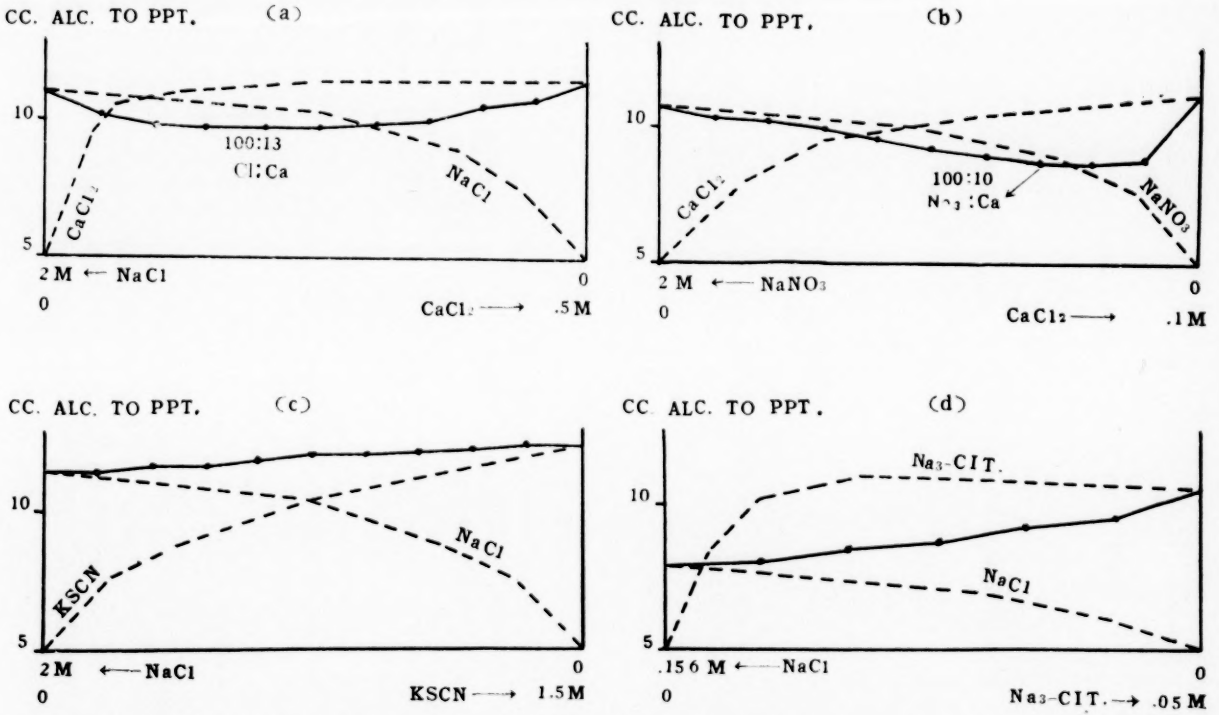

FIG. 8. The abscissæ represent various mixtures of salts (*e.g.* in (a) the middle point signifies that the gelatin contains 1 M NaCl and 0.25 M $CaCl_2$). The ordinates show the alcohol numbers of gelatin in these salt mixtures. The solid lines show the effects of the two salts combined. The dotted lines show the effects of each salt acting separately (*i.e.* at the concentration indicated on the abscissa but in the absence of the other salt), (a), (b), and (d) show antagonism. NaCl and KSCN in (c) are not antagonistic.

At the (approximate) minimum of the solid line in (a) the proportions Cl to Ca are 100: 13. In (b) the corresponding proportions of NO_3 to Ca are 100: 10. (See Table VI.)

TABLE VI.

Effect of Mixtures of Salts upon the Precipitation of Gelatin by Alcohol.

NaCl and $CaCl_2$ (antagonistic).			$NaNO_3$ and $CaCl_2$ (antagonistic).			NaCl and Na_3 citrate (antagonistic).			NaCl and KSCN (not antagonistic).		
Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.
NaCl	$CaCl_2$		$NaNO_3$	$CaCl_2$		NaCl	Na_3 in citrate.		NaCl	KSCN	
M	M	cc.	M	M	cc.	M	M	cc.	M	M	cc.
2.0	0.0	11.0	2.0	0.0	10.7	0.156	0.0	8.1	2.0	0.0	11.6
1.8	0.05	10.2	1.8	0.01	10.5	0.13	0.0083	8.4	1.8	0.15	11.6
1.6	0.1	9.9	1.6	0.02	10.3	0.105	0.0166	8.9	1.6	0.30	11.8
1.4	0.15	9.8	1.4	0.03	10.0	0.078	0.025	9.0	1.4	0.45	11.8
1.2	0.2	9.8	1.2	0.04	9.7	0.052	0.033	9.6	1.2	0.6	12.0
1.0	0.25	9.8	1.0	0.05	9.4	0.026	0.042	9.9	1.0	0.75	12.1
0.8	0.3	9.9	0.8	0.06	9.1	0.0	0.05	10.9	0.8	0.9	12.2
0.6	0.35	10.0	0.6	0.07	8.9				0.6	1.05	12.2
0.4	0.4	10.5	0.4	0.08	8.9	2 per cent gelatin A at 30°C.			0.4	1.2	12.3
0.2	0.45	10.8	0.2	0.09	9.0				0.2	1.35	12.4
0.0	0.5	11.3	0.0	0.1	11.2				0.0	1.5	12.3
3 per cent gelatin B at 26°C.			3 per cent gelatin B at 26°C.						3 per cent gelatin B at 26.5° C.		

For the effects of these salts alone (*i.e.* the dilution curves) see tables in the first paper of this series.[1] The above data, together with the dilution curves, are plotted in Fig. 8. For other details see Table I.

experiment is shown in Fig. 7. An exactly similar curve results if the NaCl is replaced by an equivalent amount of KCl, or *vice versa*.

In the second method of experimentation with combinations of salts, the concentrations of both salts are allowed to vary simultaneously. The results of these experiments are plotted in Figs. 8 and 9. In Fig. 8a, is shown the alcohol number of gelatin dissolved in various mixtures of 2 M NaCl and 0.5 M $CaCl_2$. The dotted lines show the effect of each salt separately on the alcohol number in the concentration in which it occurs in the combination. The solid line shows the effect of the combination. At the minimum of the curve, which is obviously only roughly defined, the concentrations of Cl to Ca are 100 : 13. This is

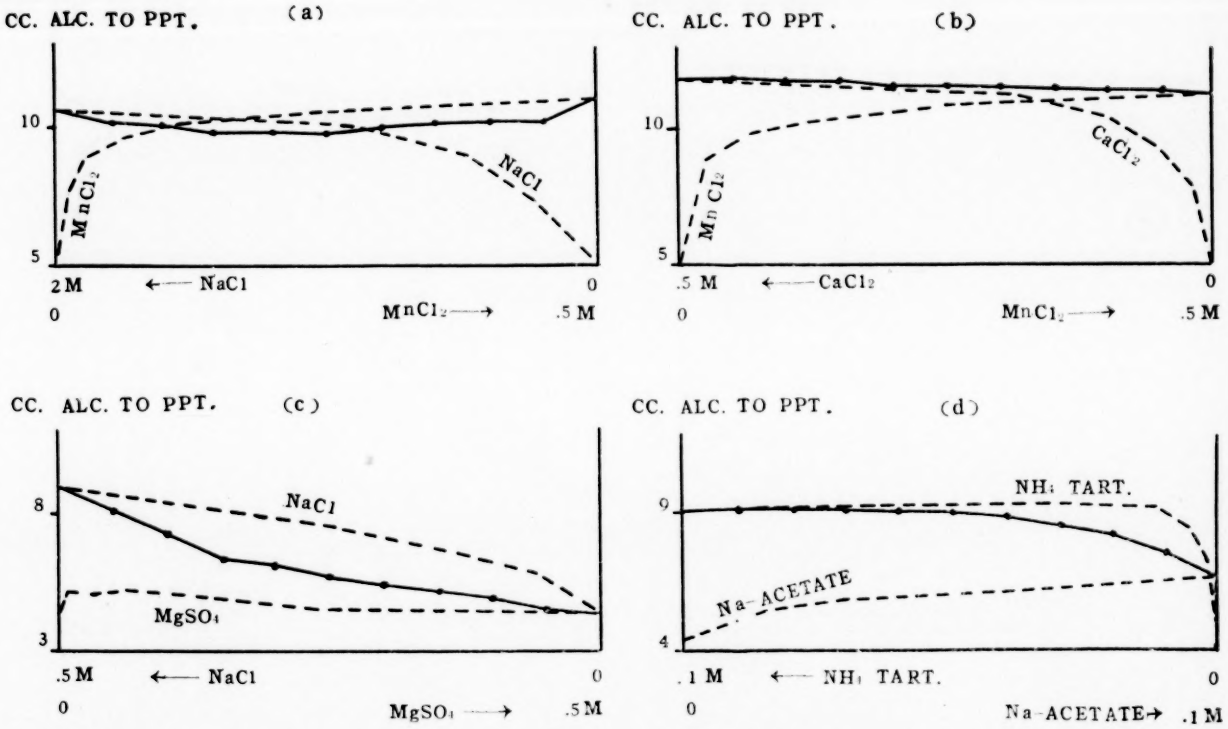

FIG. 9. The ordinates show the alcohol numbers of gelatin in various mixtures of salts which are indicated as shown on the abscissæ (*e.g.* in (a) the middle point signifies that the gelatin contains 1 M NaCl and 0.25 M $MnCl_2$). The solid lines show the combined effects. The dotted lines show the effects of the salts acting separately (*i.e.* at the concentration indicated on the abscissa but in the absence of the other salt). (a), (c), and (d) show antagonistic combinations. $CaCl_2$ and $MnCl_2$ in (b) are not antagonistic. (See Table VII.)

TABLE VII.

Effect of Mixtures of Salts on the Precipitation of Gelatin by Alcohol.

$CaCl_2$ and $MnCl_2$ (additive).			$NaCl$ and $MnCl_2$ (antagonistic).			NH_4 tartrate and Na acetate (antagonistic).			$MgSO_4$ and NaCl (antagonistic).		
Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.	Concentration of		Alcohol to precipitate.
$CaCl_2$	$MnCl_2$		$NaCl$	$MnCl_2$		NH_4 tartrate.	Na acetate.		$MgSO_4$	NaCl	
M	M	cc.	M	M	cc.	M	M	cc.	M	M	cc.
0.5	0.0	11.8	2.0	0.0	10.6	0.1	0.0	9.25	0.5	0.0	9.05
0.45	0.05	11.7	1.8	0.05	10.2	0.09	0.01	9.3	0.45	0.05	8.15
0.4	0.1	11.6	1.6	0.1	10.0	0.08	0.02	9.2	0.4	0.1	7.4
0.35	0.15	11.8	1.4	0.15	9.8	0.07	0.03	9.0	0.35	0.15	6.4
0.3	0.2	11.5	1.2	0.2	9.9	0.06	0.04	9.0	0.3	0.2	6.2
0.25	0.25	11.5	1.0	0.25	9.8	0.05	0.05	8.9	0.25	0.25	5.8
0.2	0.3	11.6	0.8	0.3	10.0	0.04	0.06	8.8	0.2	0.3	5.55
0.15	0.35	11.5	0.6	0.35	10.1	0.03	0.07	8.55	0.15	0.35	5.1
0.1	0.4	11.4	0.4	0.4	10.3	0.02	0.08	8.25	0.1	0.4	5.0
0.05	0.45	11.4	0.2	0.45	10.3	0.01	0.09	7.6	0.05	0.45	4.45
0.0	0.5	11.1	0.0	0.5	11.0	0.0	0.1	6.6	0.0	0.5	4.35

3 per cent gelatin B was used throughout. The gelatin stood over night at 26°C. before titrating.

For the effects of these salts alone (*i.e.* the dilution curves) see tables in the first paper of this series. The above data, together with the dilution curves, are plotted in Fig. 9. For other details see Table I.

of the same order of magnitude as the 100: 8 ratio found for the same salts in Fig. 1. At this point, furthermore, the alcohol number for the combination is less than that for either of the salts alone in the concentrations prevailing there. This is in accord with the fact previously demonstrated that each of these salts decreases the effect of the other on gelatin.

A similar case of antagonism between $NaNO_3$ and $CaCl_2$ is shown in Fig. 8b for $NaNO_3$ and $CaCl_2$. The proportions of NO_3 to Ca at the minimum are 100: 10. The minimum is displaced to the right because the concentration of the $CaCl_2$ used was proportionally less than in Fig. 8a. This curve disposes of the possibility that any common ion effect is concerned in these results. This being taken for granted, salts with a common ion were always used where possible, to avoid the possibility of the formation of a third salt.

Fig. 8c shows that KSCN and NaCl are not antagonistic. The graph is in this case a straight line which is at no point below the curve for either of the salts alone. A similar curve for KCl and NaCl gave likewise a straight line. In such a case as this where both salts have an equal effect on the alcohol number and where the dilution curves are alike, the additive effect can easily be determined by Osterhout's method[24,25] and would be a straight line like the one obtained experimentally. In Figs. 8a and 8b, also, there is very little error in drawing a straight line connecting the ends of the two dilution curves for the salts alone, and considering it the additive effect.

In Fig. 8d, however, the curves for Na_3 citrate and NaCl alone are so different that it is difficult to determine the additive effect. It is certain that it would not be a straight line connecting the ends of the dilution curves for such a line would fall below the curve for Na_3 citrate alone and it is evident that the additive effect of two salts on gelatin when present simultaneously but acting independently should be more than the effect of either one of them alone. The experimental curve for the combined salts shows, however, that the combined effect is less than the effect of Na_3 citrate alone. Therefore NaCl and Na_3 citrate are antagonistic.

The results of four similar experiments are plotted in Fig. 9. $MnCl_2$ and NaCl are shown to be slightly antagonistic in Fig. 9a, but $MnCl_2$ and $CaCl_2$ are not antagonistic to each other in Fig. 9b. Thus these two salts with bivalent cations are not antagonistic to each other, but they are antagonistic to salts with monovalent cations. Figs. 9c and 9d show two other cases where the additive effect is not easily determined. In both cases, however, antagonism is undoubtedly shown by the fact that the curve for the combined effect of the two salts (solid line) falls below the curve for one of the salts alone (dotted lines). For a thorough understanding of these two cases, however, other concentrations should be tried. The complete interpretation of the data here given is difficult.

Similar experiments have been tried by this method with $MgCl_2$ and Na_3 citrate, $CaCl_2$ and Na_3 citrate, $MgCl_2$ and Na_2SO_4. All these curves are strongly convex to the base line, indicating antagonism. Part of this, however, is certainly due to the formation of a third slightly soluble or slightly dissociated salt. It is at least an open question, however, whether there is not some

significance for the subject of salt antagonism in the fact that salts such as those formed in these experiments (*i.e.* $MgSO_4$, Ca citrate, and Mg citrate) have but very little effect upon the alcohol number of gelatin.

The experiment with $CaCl_2$ and Na_3 citrate has been published in a previous paper,[3] and Loeb has pointed out[23] that the precipitate obtained with alcohol at the minimum of the curve is partly due to the precipitation of Ca citrate. The gelatin is also precipitated, however, at the same concentration of alcohol. Furthermore, it would undoubtedly be possible by using weaker concentrations of the salts to obtain the effect on gelatin without the precipitation of the salt.

$MgCl_2$ is found by biologists to behave irregularly in its effects upon organisms. Similarly in gelatin it behaves neither like NaCl nor like $CaCl_2$. It has already been shown that $MgCl_2$ alone has more effect than NaCl alone, and less than $CaCl_2$ alone, on the alcohol number of gelatin.[1] A number of experiments have also been tried to determine the effects of $MgCl_2$ when present with NaCl or $CaCl_2$ in gelatin, but no satisfactory results have been obtained. When the concentrations of both salts were caused to vary simultaneously, as in the experiments just described (Figs. 8 and 9), $MgCl_2$ seems to be slightly antagonistic to both $CaCl_2$ and NaCl. When, however, the gelatin is dissolved in 0.1 or 0.5 M $MgCl_2$ and either $CaCl_2$ or NaCl is added in increasing concentrations, no decrease in the alcohol number can be observed. This would indicate that $MgCl_2$ is antagonized by neither NaCl nor $CaCl_2$. $MgCl_2$ does not behave like $CaCl_2$ when added to gelatin plus 2 M NaCl, as no decrease in the alcohol number is obtained. More experimentation on this point is necessary.

SUMMARY.

1. Monovalent salts (like NaCl) are found to be antagonistic to all salts with bivalent or trivalent anions or cations.

2. Salts with bivalent or trivalent cations are found to antagonize all salts with bivalent or trivalent anions.

3. Salts with bivalent anions (like Na_2SO_4) are found to differ from salts with bivalent cations (like $CaCl_2$) in that small con-

centrations of the latter decrease, and of the former increase, the alcohol number of gelatin plus NaCl.

4. A pair of salts with bivalent cations (*e.g.* $CaCl_2$ and $MnCl_2$) or a pair of salts with monovalent anions and cations (*e.g.* NaCl and KSCN) are not antagonistic in their effects.

NOTE ON THE RELATION OF BLOOD FAT TO SEX, AND ON THE CORRELATION BETWEEN BLOOD FAT AND EGG PRODUCTION IN THE DOMESTIC FOWL.

By OSCAR RIDDLE and J. ARTHUR HARRIS.

(*From the Station for Experimental Evolution, Carnegie Institution of Washington, Cold Spring Harbor, New York.*)

(Received for publication, November 3, 1917.)

In a recent number of this *Journal* Warner and Edmond[1] have presented a considerable series of determinations of the blood fat of White Leghorn hens and cocks. Their interesting discussion of this subject requires a few words of comment on our part.

In the treatment of their data these authors have not sufficiently pointed out the bearing of their results upon earlier work,—indeed most of the latter is ignored; the full value of their own work is not clearly brought out; and some conclusions unwarranted by their data are drawn. The purpose of the paper is stated to be "to show the relationship of blood fat of fowls to (1) egg production, (2) presence of food in the alimentary tract, (3) color of legs, etc., and (4) sex."

The clear and outstanding fact to be found in the 94 blood fat determinations made by Warner and Edmond is that *the blood of the actively laying hen contains a very disproportionately large amount of fat; that of the non-laying hen a very disproportionately small amount of fat.* This fact, however, (and others soon to be mentioned) for the blood serum of the fowl was published by Lawrence and Riddle[2] 2 months prior to the time that the samples for the above mentioned 94 blood analyses were drawn for examination. This earlier work of Lawrence and Riddle on the blood of

[1] Warner, D. E., and Edmond, H. D., *J. Biol. Chem.*, 1917, xxxi, 281.

[2] Lawrence, J. V., and Riddle, O., *Am. J. Physiol.*, 1916, xli, 430.

the fowl is not mentioned by the authors of the paper now being considered.[3]

It should be pointed out that the numerous blood fat determinations made by Warner and Edmond support and confirm the conclusions of Lawrence and Riddle, though the extent of the difference between the values for laying and non-laying hens was not anticipated by the fewer determinations made by the latter authors. Lawrence and Riddle[4] concluded as follows:

"A differentiation of the fat and phosphorus metabolism of male- and female-producing ova in the pigeon (Riddle) invites a comparison of the quantities of these substances present in the adults of the two sexes.

The blood plasma of female fowls is found to be richer in alcohol-soluble substance and phosphorus than is the plasma of the male.

The blood plasma of the sexually functioning female fowl contains more alcohol-soluble substance and more phosphorus than does the plasma of fowls with temporarily inactive ovary.

The male, the non-laying female and the actively laying female fowl are three natural groups of fowls when these are considered from the standpoint of the fat and phosphorus content of the blood plasma. The relative distribution of phosphorus in the alcohol-soluble and alcohol-insoluble fractions of the blood plasma is also different for these three groups of fowls.

The differences here observed and measured are quantitative not qualitative in kind.

These results on the fowl, together with the few data available on other forms, lend support to the view advanced by Riddle that the initial metabolic differences between eggs of different sex value persist in the organisms developed from the eggs of the two grades."

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[3] Warner and Edmond cite a short paper by Warner which appeared a month later than that of Lawrence and Riddle; the only reference in the paper under discussion to the blood fat of the fowl (from "preliminary work and involved only ten hens," and not described in their later paper) is that reproduced herewith. The italics are ours:

"A fair assumption is that the body color fades out and the blood of *high producing* hens becomes thicker, as the writer [has] shown by some recent investigations, or in other words, contains more fat and cholesterol bodies than the blood of *poor producing* hens. The average amount of fat and cholesterol bodies found in *high producing* hens is 1.426 per cent and in the *low producing* birds the average is 0.886 per cent. This work is to be published elsewhere giving a full report of the methods used." Warner, D. E., *J. Am. Assn. Instructors and Investigators Poultry Husbandry*, 1916, iii, 6.

[4] Lawrence and Riddle, *Am. J. Physiol.*, 1916, xli, 436.

Their data for sex were not carefully analyzed by Warner and Edmond. They state:[5] "There did not appear to be very much difference between the per cent of fat found in the blood of male birds and that found in the blood of females not laying." And elsewhere[6] that the average for male birds is "about the same as the per cent found in the blood of 3 year old hens." Their data largely justify this statement. But it is of importance to know, if possible, in which direction the difference lies. With this in view we have constructed the following table, in which the data are analyzed and compared with the figures for blood serum (upper rows) obtained by Lawrence and Riddle. Only the figure obtained for 3 year old hens (0.171, lowest row) is at variance with the other results of the two separate investigations, and it can be said that this figure is not a really contradictory one; for the age of the males permits a direct and proper comparison with the females which yielded an average of 0.199, but not with the much older females which gave an average of 0.171. In the two series of investigations (one on serum, the other on total blood) different methods were used; those employed by Lawrence and

TABLE I.

	Males.	Non-laying hens.	Laying hens.
Fat (serum)	155*	179	279
Phosphatides (serum)	64	74	131
Fat (blood)	0.145†	0.294§	1.175‖
" "	0.176‡	0.199¶	1.109
" "	—	0.171	—

* The method of calculation of the figures in this section of the table differs from that in the lower rows; but the proportions of blood fat in the three groups of birds is accurately shown (Lawrence and Riddle, *Am. J. Physiol.*, 1916, xli, 430).

† From Table X (Warner and Edmond, *J. Biol. Chem.*, 1917, xxxi, 292).

‡ From Table VIII (Warner and Edmond, *ibid.*, 291).

§ From Table IV (Warner and Edmond, *ibid.*, 287).

¶ From Table II (Warner and Edmond, *ibid.*, 285); these were much older birds, and "had about completed their 3rd year of laying."

‖ From Table III (Warner and Edmond, *ibid.*, 285).

[5] Warner and Edmond, *J. Biol. Chem.*, 1917, xxxi, 293.

[6] Warner and Edmond, *ibid.*, 292.

Riddle utilized very much larger quantities of blood and probably the error involved in the individual determinations was thus reduced. The later and larger series of blood fat determinations therefore confirms the results obtained by Lawrence and Riddle.

Most readers of Warner and Edmond's paper will certainly get the impression that the blood of 1 year old hens has an average fat value of 0.407 per cent, while that of 3 year old hens has a value of only 0.171 per cent. The actual relation of these two groups is as 0.199 to 0.171. The group of 3 year old hens which gave an average of 0.171 were all non-layers and only non-layers among the 1 year old hens may properly be compared with them.

It may next be pointed out that the data "on the presence of food in the alimentary tract" do not justify the conclusion drawn, nor indeed any conclusion whatsoever. The following statement is made:[7] "It has been reported by Mathews that in man and animals the blood is much richer in fat after they have been eating than it is after fasting. This is not true with hens, as shown from Table VIII. . . . . The [small] difference . . . . would indicate that fasting for 16 hours has no effect upon the amount of fat in the blood." In reality, 16 hours is not a fasting period in the fowl. It more nearly represents the length of time between meals, at least on many of the very short days of winter; and certainly there is some food still in the alimentary tract of a fowl for more than 16 hours after feeding.

There are, however, two reasons why the data cannot answer the question of the relative amount of fat in the blood soon after, as compared with 16 hours after a meal. These are, first, that the *female* fowl was used, and in these birds the individual variation is enormous, the really decisive factor associated with blood fat values being the "laying" or "non-laying" condition of the hen. Other figures by these authors show that the blood fat of a laying hen (1.953) may be more than twenty times greater than that of a non-laying hen (0.083). Their figures (Table VI) also indicate that this value in laying hens may vary between 0.258 and 1.953. It is therefore obvious that one may not expect to find *differences due to fasting for 16 hours* in the averages obtained

[7] Warner and Edmond, *ibid.*, 289.

from twelve individuals of one group when compared with twelve individuals of the other group. An illustration of the unsuitability of the data for this purpose is the following: If the twelfth (last) bird in each series be omitted from the averages, these latter are changed from 0.405 and 0.396 to 0.268 and 0.263 respectively. Only in males, in which the fluctuations of the blood fat values are much decreased, could one hope for success in a study of this question in the fowl. Still another reason for the inadmissibility of the data as submitted is that no basis for the selection of the particular individuals chosen and compared is given. As the data stand, and if one is permitted to select at will the "non-fasting" birds from the whole group of fat determinations, it is possible to have those birds show *more* or show *less* blood fat than the "fasting" birds. These data, therefore, supply no evidence that the fowl is an exception to the well known rule that the per cent of fat in the blood increases soon after the ingestion of food. In all of the fat determinations made by Lawrence and Riddle this fact was recognized, and the blood samples were all drawn at approximately the same time of day; namely, in the early afternoon.

Our next point concerns the correlation between the fat content of the blood and egg production. Admitting that birds which are laying at the time the blood samples are taken differ from those which are not laying, a further problem arises concerning the relationship of the percentage of fat found in the blood and the total egg record of the bird for the year.

These authors say:[8] "The fat content of the blood is correlated with egg laying activity, and *there is also a slight correlation between the amount of fat in the blood and high yearly production.*"

But in their summary, they conclude:[9] "*There is little or no correlation between the amount of fat in the hen's blood and her yearly egg yield.* On the other hand the blood of a hen laying at the time the sample is taken is much richer in fat than that of a hen which is not laying."[10]

Now both of these contradictory statements cannot be true. There is, indeed, no reason why statements concerning correlation

[8] Warner and Edmond, *ibid.*, 288.

[9] Warner and Edmond, *ibid.*, 293.

[10] The italics are ours in both quotations.

should be of a general and vague nature. Correlation is a very definite quantitative phenomenon, measurable on a universally applicable scale of -1 to $+1$. The numerical value of the correlation coefficient, r, may be determined in any instance for two measured variables, such for example, as fat content and annual egg record, by very simple and well known formulas.

We now turn to the actual results which may be obtained by applying the correlation formulas[11] to the data published by Warner and Edmond. Using a simple method of direct summation of the actual values, their squares and their products,[12] we find the following results for the whole series of 1 year old hens:[13]

$$r_{fe} = +0.247 \pm 0.076$$

Thus, taking the whole series of 1 year old birds examined, the hens which have a larger amount of fat in their blood in October have, on the average, laid a larger number of eggs during the year.

[11] Warner and Edmond must have been fully acquainted with the advantages of applying the correlation formulas to such problems as these, for the data contained in the preliminary papers by Blakeslee and Warner, cited and discussed by Warner and Edmond, together with far more extensive data collected since these two preliminary papers were published, have been carefully treated in great detail by the statistical methods in a paper by Harris, Blakeslee, and Warner (*Genetics*, 1917, ii, 36; *Proc. Nat. Acad. Sc.*, 1917, iii, 237). These papers, like that by Lawrence and Riddle, they have, quite inadvertently, failed to cite, although they contain much that throws light upon the problems considered by them. A further investigation of the problem of the interrelationship between egg laying activity at various periods which also contains materials bearing very directly upon the problem of the physiology of egg production is now in press (*Genetics*, 1918, iii, 27).

[12] Harris, J. A., *Am. Naturalist*, 1910, xliv, 693.

[13] Those unfamiliar with statistical formulas need only remember that the correlation coefficient measures the intensity of interrelationships between two variables on the scale of -1 to $+1$. Thus if annual egg production is lower in birds with lower percentages of fat and higher in birds with higher percentages of fat correlation between egg production and fat content is positive in sign and lies somewhere between no correlation and perfect correlation. If high annual egg record is associated with low fat content and low annual record with high fat content, correlation is negative in sign and is measured by a coefficient lying between 0 and perfect negative correlation.

But this group is highly heterogeneous. It comprises birds which were still laying at the time the blood samples were taken and those which had ceased to lay.[14] Dividing the birds into two groups on the basis of their laying activity at the time of sampling we find the following values for averages, variabilities, and correlations.

For 54 birds which had ceased laying,

$$\bar{f} = 0.199 \qquad \bar{e} = 139.09$$
$$\sigma_f = 0.0847 \qquad \sigma_e = 38.948$$
$$V_f = 42.36 \qquad V_e = 28.00$$
$$r_{fe} = -0.296 \pm 0.084$$

For 16 birds which were still laying at the time the samples were taken,

$$\bar{f} = 1.103 \qquad \bar{e} = 163.37$$
$$\sigma_f = 0.6102 \qquad \sigma_e = 30.503$$
$$V_f = 55.30 \qquad V_e = 18.67$$
$$r_{fe} = +0.351 \pm 0.147$$

Here f = percentage fat, e = number of eggs laid during the year, the bars over these letters denote the averages, the sigmas their absolute variabilities in terms of square root of mean square deviation from their mean, V their coefficients of variation, *i.e.*, $100\sigma/$ mean, and r the correlation between them.

Now while the probable errors of these correlations are large, because of the relatively few determinations, the correlations suggest at once that *while the birds are laying* those which have the highest percentages of blood fat are those which make the highest egg records, but that *after the bird has ceased laying in the autumn* those which have laid the greatest number of eggs during the year have the blood most depleted of fat; or, at any rate, have blood with least fat.

Support for this view is furnished by splitting the birds up into two groups according to the color of the beak, legs, and vent—a character which when taken in October may be taken as a criterion of annual egg production.[15]

[14] One bird had not laid at all during the year.

[15] Blakeslee and Warner, as cited by Warner and Edmonds. See also Harris, Blakeslee, and Warner, *Genetics*, 1917, ii, 36; *Proc. Nat. Acad. Sc.*, 1917, iii, 237. Blakeslee, Harris, Warner, and Kirkpatrick, *Storrs Agric. Exp. Station, Bull. 92*, 1917.

Birds with yellow beaks, legs, and vent, N = 23

$$r_{fe} = 0.411 \pm - 0.117^*$$

Birds with pale beaks, legs, and vent, N = 18

$$r_{fe} = + 0.532 \pm 0.114$$

* This is the value obtained by using only the twenty-three 1 year old birds included in Table VII of Warner and Edmond. If the nine 3 year old birds which they have lumped with these are included the correlation is $r = - 0.332 \pm 0.106$. The correlation for the nine 3 year old birds alone is $r = - 0.021 \pm 0.225$.

The results are the same in sign but numerically larger than the coefficients based on the actual record concerning laying activity. They point to the same conclusion; namely, that while birds are in the actively laying condition there is a *positive* correlation between annual egg record and per cent of fat in the blood, but that *after the egg-producing capacity of the bird has been exhausted*, there is a *negative* correlation between egg record and the amount of fat in the blood.

If this is true, one might expect the correlation to change with the lapse of time since laying. This point may be tested by splitting the data of Warner and Edmond into groups according to the number of days since laying to the time the blood sample was taken, and determining the correlation between fat content and egg production in each of these groups separately. We find:

For birds laying at time blood sample was taken, N = 16

$$r_{fe} = + 0.351 \pm 0.148$$

For birds which had not laid for from 1 to 24 days, N = 16

$$r_{fe} = + 0.054 \pm 0.168$$

For birds which had not laid for from 25 to 29 days, N = 22

$$r_{fe} = - 0.132 \pm 0.141$$

For birds which had not laid for from 60 to 365 days, N = 16

$$r_{fe} = - 0.620 \pm 0.103$$

Thus, as suggested above, there is a progressive change in the nature of the correlation between fat content and total egg records which is positive for birds in a laying condition, sinks to zero after the cessation of laying, and finally takes a high negative value in birds which have long since ceased to lay.

Summarizing these statements on correlation we note: At the end of the first laying year, in October, the correlation between the per cent of fat in the blood and the annual egg record of the year is positive in the case of birds which are still laying, but negative in the case of birds which have ceased laying; or, in other words, birds which have laid larger numbers of eggs and are still laying have a higher percentage of fat in their blood than laying birds which have made a poor record during the year. Birds which have laid a large number of eggs, but have exhausted their fertility, have a smaller per cent of fat in their blood than nonlaying birds which have made poor egg records.

By dividing records into classes, according to the number of days since the cessation of laying, the correlation between October blood fat content and annual egg record changes systematically from a positive to a negative relationship.

This brief discussion will perhaps indicate how useless it is to draw conclusions concerning correlations between blood fat and annual egg production without actually determining the correlation coefficients. The constants given here are of course subject to the limitations of the series of data upon which they are based. Larger sets of determinations, made at various times of the year, when analyzed by proper statistical formulas, should yield further important information on the relation of fat metabolism to the functioning of the ovary.

Addendum.—In the foregoing treatment of the data published by Warner and Edmond[1] we had two purposes: (*a*) to call the attention of biological chemists to earlier work on the same subject which those authors entirely ignored, and (*b*) to clear up some matters concerning the interpretation of the data presented by them. We regret, therefore, that their second communication[16] not only fails to contribute to these desirable ends but introduces other errors, some misleading quotations and statements, and much wholly irrelevant discussion.

It therefore seems quite useless, as well as personally undesirable to us, to continue this discussion by commenting further on their second publication. For the convenience of the reader who may, if he cares to take the time, verify the statements from the original papers, we may add this summary.

1. A note by Warner and Edmond in a poultry publication appeared a month after the work of Lawrence and Riddle was published. It dealt

[1] Warner and Edmond, *J. Biol. Chem.*, 1918, xxxiv, 171.

with methods of selecting high producing and low producing hens, and their only reference to the problem of blood fat in the fowl has been fully quoted by us.[3] Warner and Edmond published nothing on blood fat in relation to *sex* nor in relation to *laying* and *non-laying* until 11 months after the paper by Lawrence and Riddle appeared, which dealt exclusively with these problems and not with that of high and low production. Their samples for this purpose were not drawn until 2 months after this publication.

2. The data of Warner and Edmond when properly classified and analyzed confirm the conclusions of Lawrence and Riddle in so far as the problem of sex and of laying and the non-laying condition of the bird is concerned.

3. The data of Warner and Edmond are not sufficiently extensive to decide finally the question of the possible relationship between percentage of blood fat and high and low production. When properly classified biologically and adequately analyzed statistically they indicate the incorrectness of the conclusion of Warner and Edmond[17] that, "There is little or no correlation between the amount of fat in the hen's blood and her yearly egg yield."

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[17] Warner and Edmond, *J. Biol. Chem.*, 1917, xxxi, 293.

BLOOD FAT IN FOWLS.

By D. E. WARNER and H. D. EDMOND.

(*From the Poultry and Chemical Laboratories of the Connecticut Agricultural Experiment Station, Storrs.*)

(Received for publication, January 27, 1918.)

In another article in this issue, Riddle and Harris have commented on a previous paper by us,[1] and our work has been criticized for ignoring what has been termed earlier work on the subject by Lawrence and Riddle. Riddle and Harris maintain that the work of Lawrence and Riddle was published prior to our work and that they pointed out that the blood of actively laying hens and hens which were laying was richer in fat and phosphorus than that of hens not laying. It is true that Lawrence and Riddle's paper[2] was published 1 month prior to the appearance of our paper in October, 1916.[3] It must be pointed out, however, in this connection, that Blakeslee and Warner, as early as September and October, 1914, realized that there was a relation between the egg laying activity and pigmentation in the domestic fowl and they published data covering these points.[4] Blakeslee and Warner's work has been further elaborated by Harris, Blakeslee, and Warner.[5] The reason given by Blakeslee and Warner for the pigment changing from a yellow to a pale color in the laying hen was that "the most natural assumption is that laying removes yellow with the egg yolks more rapidly than it can be replaced by the normal metabolism, and in consequence, the earlobes, beak, and the legs become pale by this subtraction of

[1] Warner, D. E., and Edmond, H. D., *J. Biol. Chem.*, 1917, xxxi, 281.

[2] Lawrence, J. V., and Riddle, O., *Am. J. Physiol.*, 1916, xli, 430.

[3] Warner, D. E., *J. Am. Assn. Instructors and Investigators Poultry Husbandry*, 1916, iii, 6.

[4] Blakeslee, A. F., and Warner, D. E., *Science*, 1915, xli, 432; *Am. Naturalist*, 1915, xlix, 360.

[5] Harris, J. R., Blakeslee, A. E., and Warner, D. E., *Genetics*, 1917, ii, 36. Harris, J. A., Blakeslee, A. F., and Kirkpatrick, W. F., *Proc. Nat. Acad. Sc.*, 1917, iii, 565. Blakeslee, Harris, Warner, and Kirkpatrick, *Storrs Agric. Exp. Station, Bull 92*, 1917.

pigment."[4] Furthermore, the short paper to which Riddle and Harris have referred and which was published in October, 1916,[3] was, as a matter of fact, read on August 2, 1916 (a month earlier than the publication of Lawrence and Riddle's paper), before the American Association of Poultry Instructors and Investigators in its regular annual meeting, held that year at the University of Ohio. The presentation of a paper at a meeting of this character is presumed to have established priority in precisely the same way as its publication in the organization's official journal. This statement explains why that paper of Lawrence and Riddle was not alluded to as "earlier work."

There is, however, another and more important reason for not seriously considering the results of their experiments. A casual examination of the following table[6] from their paper suggests to our minds that the work does not fulfil the requirements for carefully controlled experimental study.

TABLE I.

Analyses of Blood Plasma from Males, Non-Laying, and Laying Female Fowls.

Males.

Date.	Description.	Weight.
Apr. 11	White Wyandotte (mongrel) 2 years old	2,282
" 21	" " " 2 " "	2,410
Nov. 23	Mongrel	2,480
	Non-laying females.	
Dec. 2	White Wyandotte 1½ years old	1,405
" 18	Houdan 1½ years old	1,375
" 18	Wyandotte-Houdan (mongrel)? 3 years?	2,015
	Females laying eggs.	
Apr. 5	Game, 3–4 years old	1,750
" 5	Plymouth Rock 3 years old	1,500
" 11	White Wyandotte (mongrel) 1 year old	1,404
" 11	" " " 1 " "	1,332
" 21	Wyandotte-Houdan (mongrel) 11 months old	1,380

It will be noted that Lawrence and Riddle used a total of only eight females and that these were a mixed lot, consisting of Games, Houdans, Rocks, Wyandottes, Wyandotte mongrels, and crosses.

[6] Lawrence and Riddle, *Am. J. Physiol.*, 1916, xli, 433 (parts of the table are omitted).

The birds were not only decidedly heterogeneous as to breed, but ranged in age from 11 months to 4 years. Furthermore, blood samples were drawn from the non-layers in December, one of the most unfavorable months of the year for egg production, whereas samples from the layers were drawn in April, or at the natural laying season. If we may be permitted at this point to conjecture, we would surmise that if all the samples had been taken in either December or in April, the results would have been radically different. Probably all the birds would have been laying in April and it is certain that no December eggs could reasonably be expected from 3 year old Plymouth Rocks and 4 year old Games. Lastly, Lawrence and Riddle have not clearly stated the several ages of their birds. The table shows that of their five layers three were 1 year old or less, and yet they state:[7] "The egg laying history (for one or more years) of the birds used was known from their trap-nest records. The ovary of every female killed was also examined to make certain the distinction between the laying and non-laying fowls. In every case the findings in the ovary and the trap-nest records are in agreement." Their birds must certainly have laid at a much earlier age than any we have ever handled.

Riddle and Harris have taken exception to the discussion and conclusions drawn from Table VIII, in which we have stated:[8] "It has been reported by Mathews that in man and animals, the blood is much richer in fat after they have been eating, than in fasting. This is not true with hens, as shown from Table VIII. . . . . The [small] difference would indicate that fasting for 16 hours has no effect upon the amount of fat in the blood." It may be true that the word "fasting" was ill chosen, because the term "fasting" might mean almost anything from going without food for a few hours to going without it for a period producing symptoms of starvation. It is doubtful if Mathews meant by fasting an absence of food in the alimentary tract. The point that we intended to bring out was the fact that with the hens in the test given, the eating of food just a short time (not more than 2 hours) before the sampling of the blood, had no appreciable effect on the fat content. Emphasis must be placed on the effect of ingested food after a period without food. This view is con-

[7] Lawrence and Riddle, *Am. J. Physiol.*, 1916, xli, 431.

[8] Warner and Edmond, *J. Biol. Chem.*, 1917, xxxi, 289.

firmed by the last part of the statement made by Riddle and Harris when they state that our "data, therefore, supply no evidence that fowls are an exception to the well known rule that the per cent of fat in the blood increases soon after the ingestion of food." Here we find that the emphasis is not put upon the period of going without food, but upon the period immediately following the taking of food, when they say "soon" after the ingestion of food.

Riddle and Harris also point out that the great variation between laying and non-laying, 0.258 and 1.953 per cent, must invalidate our conclusion, but when they give the figures with the last bird in each group omitted, the results are practically the same in proportion. Before the change was made, the per cents for fasted and non-fasted are 0.405 and 0.396, a difference of 0.009, and after the change, 0.268 and 0.263 respectively, a difference of only 0.005, both differences being in favor of the same group and very small in each case.

Riddle and Harris have stated that it is idle "to draw conclusions concerning correlations between the blood fat and annual egg production *without actually determining the correlation coefficients.*"[9] As a matter of fact, correlation coefficients were worked out by the writers and discussed at several meetings. We still maintain that the correlation coefficients do not show that one can pick out the high producing hens from the fat content of the blood as a whole. Consequently, we stated that there is little or no correlation between the amount of fat in a hen's blood and her yearly egg production. This conclusion was based on the theory that r must be at least 0.30 and not less than six times the size of the probable error, otherwise the correlation cannot be considered at all marked. We believe that Riddle and Harris confirm the above statement when they show, by using our figures, that the negative correlation for the non-layers was greater than the positive correlation for the whole series of 1 year old hens. Their results tend to show that unless there happened to be some hens laying at the time the blood samples were taken (sixteen hens were laying in this case) there is a definite negative correlation.

In conclusion, the fat content of the blood as a whole *does* provide us with evidence as to whether a hen is laying at any particular time, but not as to whether she is a high or low producing hen.

[9] The italics are our own.

LECITHIN. II.

PREPARATION OF PURE LECITHIN; COMPOSITION AND STABILITY OF LECITHIN CADMIUM CHLORIDE.

By P. A. LEVENE and C. J. WEST.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 9, 1918.)

The preparation of pure lecithin has acquired in recent years an interest other than merely academic. The physiological action of lecithin has been studied from more than one point of view by several investigators. Yet it is safe to suspect that not one of them had the assurance of the purity of the material employed by him. Indeed, as it has been stated before, there is no record in the chemical literature on lecithin of a substance which answers all the requirements of a pure lecithin.

There exists a unanimity of opinion among workers on lecithin that cadmium chloride is the most serviceable reagent for the purification of the substance. This reagent was introduced many years ago and was more recently reintroduced for preparation of pure lecithin by Bergell.[1] However, at the time of Bergell's work it was not yet known that a sample of lecithin may possess the analytical values required by the theory for the pure substance and yet contain an appreciable proportion of cephalin.

The discovery that the base contained in cephalin was aminoethanol, indicated the way by which even small admixtures of cephalin could be detected in a sample of lecithin, the basic component of which is choline. The introduction by Van Slyke of a convenient method for amino nitrogen estimation has made the test for the presence of cephalin in a given sample of lecithin a comparatively easy task. Taking cognizance of this recently acquired information Maclean[2] made a new attempt to purify

[1] Bergell, P., *Ber. chem. Ges.*, 1900, xxxiii, 2584.

[2] Maclean, H., *Biochem. J.*, 1915, ix, 351.

lecithin. He too made use of cadmium chloride for the precipitation of the phosphatide. By a series of purifications he arrived at a sample of the substance which was free from amino nitrogen, and which he considered a pure lecithin cadmium chloride salt. He also reached the conclusion that crude lecithin may be brought to a state of absolute purity by his procedure. The work of Maclean marks a great progress in the chemistry of lecithin. Unfortunately, on the one hand Maclean did not give complete analytical data of his purified material, and on the other hand very ingenious and careful workers such as Thudichum and later Erlandsen have found that samples of lecithin cadmium chloride, when reconverted into free lecithin, furnished material which no longer possessed the composition required by the theory for the pure substance. The conclusion of these workers was that in the process of liberation from the cadmium chloride salt, lecithin suffered partial decomposition. Indeed such a change might take place without having a marked influence on the numerical values of the nitrogen and phosphorus, since the proportion of these in lecithin is rather small.

Hence, the problem of lecithin as it presented itself to us in its latest phase was: First, is it possible to purify crude lecithin by means of cadmium chloride? Second, is it possible to reconvert a lecithin cadmium salt into free lecithin without its partial decomposition?

The experience in this laboratory in handling various tissue elements has brought the conviction that the majority of them did not possess the great lability ascribed to them by the earlier workers. In fact the greatest difficulty in unraveling the structure of these substances lay not in their lability but in their too great resistance to the action of degrading reagents. Hence, at the very outset we were inclined to accept the correctness of the observations of Thudichum[3] and of Erlandsen[4] as to the difficulty of obtaining an analytically true lecithin from a cadmium salt possessing correct analytical data. We were not ready to accept the interpretations offered by these workers. It seemed to us possible that the alteration in the elementary composition

[3] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884.

[4] Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 116.

might have been brought about not by a cleavage but by a chemical alteration of the original material. Bearing in mind that cephalin is a frequent admixture of lecithin, and bearing in mind the readiness with which the latter undergoes alteration, we made the following assumption. The original cadmium salt in the hands of Thudichum and of Erlandsen contained an admixture of cephalin. In the course of the prolonged manipulations required for the removal of the cadmium chloride the cephalin undergoes alteration, which then affects the elementary composition of the material in the direction observed by the previous workers. This assumption was fully sustained by experiment.

Two samples of lecithin cadmium chloride were employed for the work. One contained 7.6 per cent of its nitrogen in form of amino nitrogen, the other was entirely free from amino nitrogen. Both were freed from cadmium chloride in the same manner. For convenience of analysis the two samples of free lecithin were converted into their dihydro derivatives. The first sample showed a carbon content of 63.3 per cent instead of 65.3 required by theory, whereas the second sample possessed correct analytical values. Thus it was conclusively demonstrated that it was possible to obtain pure free lecithin from its pure cadmium chloride salt.

The second part of the problem, dealing with the extent of purification which can be accomplished by means of cadmium chloride, was solved in the following manner. The alcoholic extract of egg yolk or of yolk powder served as starting material. One sample of lecithin was prepared from the phosphatides precipitated by means of acetone. The other was obtained by precipitating with a solution of cadmium chloride from the egg oil (mother liquor from the phosphatides, which was liberated from acetone). The former samples contained 7.6 per cent, the latter about 2 per cent of its nitrogen in the form of amino nitrogen. The first sample was then purified, following exactly the directions of Maclean, and led to a sample of cadmium salt which possessed 6.7 per cent of its nitrogen in form of amino nitrogen. The second was purified by recrystallization from ethyl acetate-ethyl alcohol and furnished a cadmium salt free from impurities.

Thus it seemed that samples of lecithin which could be freed from cephalin rapidly, before the latter underwent oxidation, led

to analytically pure lecithin through recrystallization of its cadmium chloride salt; samples which contained a high proportion of cephalin could not be purified by means of the cadmium process.

SUMMARY.

For the preparation of free analytically pure lecithin the following procedure is recommended:

Precipitate lecithin from egg oil by means of a solution of cadmium chloride.

Purify the salt by recrystallization from ethyl acetate until free from amino nitrogen.

Liberate lecithin from the cadmium salt by means of ammonium carbonate.

The details of the procedure are given in the experimental part.

EXPERIMENTAL.

Cadmium Chloride Compound.

Composition of the Salt of Cadmium Chloride and Lecithin.—The composition of the cadmium chloride compound of lecithin has been reported by various investigators as follows:

	C	H	N	P	Cd
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Bergell				3.01	12.62
Thudichum			1.34	3.28	
Erlandsen	45.66	7.07	1.46	3.19	17.54
Strecker[5]	50.75	8.38			13.07
Ulpiani and Selli[6]	50.16	8.29	1.37	3.09	13.93
Heubner[7]				3.50	14.9
Eppler[8]	46.41	7.53	1.46	2.95	17.46
	47.42	7.65	1.50	3.01	16.29

Our experience has shown that this salt is of variable composition, depending upon the character of the lecithin used for its

[5] Strecker, A., *Ann. Chem.*, 1868, cxlviii, 77.
[6] Ulpiani, C., and Selli, G., *Gaz. chim. ital.*, 1902, xxxii, 466.
[7] Heubner, W., *Arch. exp. Path. u. Pharm.*, 1908, lix, 420.
[8] Eppler, J., *Z. physiol. Chem.*, 1913, lxxxvii, 243.

preparation. A few of the many samples analyzed are reported below.

Primary Alcoholic Extract.—The cadmium chloride compound was prepared in the usual manner: An alcoholic solution of lecithin was precipitated with an excess of an alcoholic solution of cadmium chloride, saturated at room temperature. The precipitate, which soon settled out, was washed thoroughly with 95 per cent and then with absolute alcohol, either by decantation or on a filter, and finally with dry acetone. The dry salt was recrystallized from a mixture of 2 parts ethyl acetate and 1 part 80 per cent ethyl alcohol.[9, 10] The composition of the salt was variable, as the following analyses show:

Sample No.	C	H	N	P	Cd
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
321	49.24	7.87	1.73	3.49	
323	51.73	8.08	1.68	3.23	
336	50.30	8.09	1.55	3.18	
338	49.26	7.68	1.52		
341			1.52	3.07	
352			1.71	3.16	
533			1.44	2.92	
534			1.67	3.18	
458			1.75	3.15	12.73

Primary Ethereal Extract.—The primary ethereal extract was separated in the usual way and the lecithin fraction prepared as above:

Sample No.	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
374	49.45	7.88	1.37	2.90

Secondary Alcoholic Extract.—Samples of the cadmium salt prepared in the usual manner from the lecithin fraction of the secondary alcoholic extract showed nearly the same composition as the above.

[9] Willstätter, R., and Lüdecke, K., *Ber. chem. Ges.*, 1904, xxxvii, 3753.
[10] Maclean, *Biochem. J.*, 1915, ix, 370.

Sample No.	C	H	N	P	Cd
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
353	50.37	7.23	1.55	3.08	
369	50.43		1.50	3.15	13.10
378	48.25	7.74	1.45	2.97	

Mother Liquor of Cadmium Salt.—The alcoholic mother liquor from the first precipitation of the cadmium chloride compound, upon concentration, gave a thick sirup, soluble in alcohol, from which acetone precipitated a gum-like substance. The sirup, diluted with alcohol, gave an abundant precipitate with alcoholic cadmium chloride. This had a composition similar to the first precipitate.

Sample No.	N	P	Cd	NH_2-N
	per cent	*per cent*	*per cent*	*per cent*
503	1.65	3.17	13.01	11.00

Cadmium Salt from Egg Oil.—In view of the results of Fränkel and Bolaffio,[11] who claim to have isolated a phosphatide, as the cadmium salt, from the acetone-soluble fraction of egg yolk, which contained 1.84 per cent N and 0.51 per cent P, or a ratio of N: P = 8: 1, it was of interest to determine the composition of the phosphorus-containing lipoids which remain dissolved in the egg fat, extracted by means of acetone. The following fractions of the acetone extract were prepared:

A. The concentrated acetone extract (egg oil), a semisolid mass at 0° (but which could not be filtered at 0°), was extracted with acetone, thus removing a considerable amount of acetone-insoluble lipoids. The acetone solution was concentrated and the resulting oil extracted with a large volume of 95 per cent alcohol at room temperature (I). The residue was again extracted with alcohol at room temperature (II). A third extraction removed little material which was precipitated by cadmium chloride and was therefore discarded.

B. Another sample of the same oil was first extracted with ether, in order that any water-soluble nitrogen-containing compounds might thus be removed (they would remain in the aqueous por-

[11] Fränkel, S., and Bolaffio, C., *Biochem. Z.*, 1908, ix, 45.

tion). The ether was removed on the steam bath and the resulting oil extracted with alcohol as before (III).

C. A third experiment started with an ethereal solution of egg lipoids, prepared by extracting fresh egg yolk with ether and concentrating the solution. This extract was precipitated with acetone, the acetone-ether solution concentrated, and the resulting oil extracted with alcohol (IV).

These four fractions were then precipitated with alcoholic cadmium chloride solution, and the salt was purified in the usual way. Analyses do not indicate a compound with N: P = 8: 1, but rather a lecithin-like body.

Fraction.	Sample No.	C	H	N	P	Cd	NH_2-N
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	446			1.41	2.93		
	453			1.43	3.05	10.75	2.4
II	445			1.41	2.65		
III	461			1.41	2.93	10.65	
	505			1.41	3.00		1.4
IV	374	49.45	7.88	1.37	2.90		
	377			1.37	3.04	13.60	

Optical Activity of the Cadmium Chloride Salt.—After trying many solvents, it was found that the cadmium chloride salt could be dissolved in boiling xylene, and that this solution, on cooling, could be used for determining the optical activity of the salt. Ulpiani and Willstätter and Lüdecke have shown that the activity of the glycerophosphoric acid of lecithin varies with the manner of the preparation of the material. Therefore the values obtained for the salt, which varied from $[\alpha]_D^{20} = +2.0°$ to $+ 4.25°$, are not to be considered absolute values, nor are the variations due to impurities. A comparison of the amino nitrogen content and the optical activity shows that the content of amino nitrogen does not influence the rotation of the salt. The same appears to be true of the hydrolecithin.

Recrystallization of the Cadmium Salt.—Results of various investigators, especially Heubner, would indicate that the cad-

mium chloride salt was unstable, and that it could not be recrystallized without marked decomposition. To determine this several samples were recrystallized repeatedly, the N and P contents being determined for each sample. Our results indicate that the salt is stable under these conditions.

Sample No.	C	H	N	P	Cd
			Series I.		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
323			1.68	3.23	
336			1.55	3.18	
342			1.61	3.16	
			Series II.		
458			1.75	3.15	12.73
459			1.81	3.07	13.53
462			1.70	3.12	
463			1.63	3.22	13.36
493			1.64	3.13	14.02
			Series III.		
478	48.57	8.58	1.84	2.87	12.65
491			1.84	2.97	12.40
495			1.83	3.11	12.38
502	48.49	8.44	1.77	3.05	13.42
			Series IV.		
152		48.12	7.70	1.40	2.93
171		47.88	7.79	1.46	3.09
179		48.12	7.70	1.55	
213		47.78	7.68		

The mother liquor of the various fractions on concentration gave an ether-soluble substance, which could be precipitated by pouring into absolute alcohol. Upon analysis it showed the following composition:

Sample No.	N *per cent*	P *per cent*
504	1.65	2.95
516	1.70	2.61

Ether Extraction of the Cadmium Salt.—The finely powdered cadmium compound was suspended in 6 parts of ether and allowed to stand over night. The following morning the ether was decanted as far as possible and the insoluble salt filtered off and recrystallized from ethyl acetate-ethyl alcohol mixture.

Sample No.	C *per cent*	H *per cent*	N *per cent*	P *per cent*	Cd *per cent*
349	50.17	7.81	1.54	2.62	12.40
351	49.39	7.46	1.52	3.25	

A second experiment was made with the cadmium salt from the secondary alcoholic extract, using first ordinary ether (a) and then extracting with moist ether (b).

Sample No.	C *per cent*	H *per cent*	N *per cent*	P *per cent*	Cd *per cent*	NH_3-N *per cent*
(a) 409			1.49	3.11		
(b) 422	49.85	8.08	1.55	2.90	14.91	1.6

Decomposition of Cadmium Salt with Ammonium Carbonate.—The first question we attempted to answer was whether we could decompose the cadmium chloride compound with ammonium carbonate, according to Bergell, and obtain from the decomposition product a cadmium chloride salt with the same composition as the original.

25 gm. of recrystallized cadmium chloride compound (No. 2) were suspended in 250 cc. of 80 per cent alcohol, the alcohol was heated to boiling, and the mixture treated with finely powdered ammonium carbonate until the boiling mixture was alkaline to litmus. The cooled alcoholic solution was filtered, concentrated *in vacuo*, the residue taken up in alcohol, and precipitated with alcoholic cadmium chloride solution. The salt which separated was recrystallized from ethyl acetate-ethyl alcohol (No. 4).

A second sample (No. 234) was decomposed in the same manner, but before precipitating with cadmium chloride the lecithin was washed with water and precipitated with acetone (No. 235).

Sample No.	C	H	N	P	NH_2-N
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
2	47.35	8.05	1.53	3.25	
4	47.95	8.33	1.62	2.31	
30	46.85	7.94			
116	47.92	7.82			
234	48.61	7.88	1.67	2.88	
235	48.91	7.99			
237	48.99	7.93			7.6
243	47.36	7.59			6.7

The last experiment was carried out in order to determine whether the method recommended by Maclean would give us an amino-free product.

100 gm. of Sample 234 were suspended in 900 cc. of ether containing a trace of alcohol, and allowed to stand over night. The ether was then decanted as completely as possible and a fresh lot of ether added. The following day the material was filtered off and recrystallized from ethyl acetate and ethyl alcohol. This product was suspended in boiling 80 per cent alcohol and decomposed with ammonium carbonate. The filtered alcoholic solution was concentrated, the lecithin washed with water, and the alcoholic solution precipitated with cadmium chloride. Analysis of the amino content showed that the product contained nearly as much cephalin as the original material (7.6 and 6.7 per cent amino nitrogen).

The same material was used to determine whether or not recrystallization from ethyl acetate and ethyl alcohol would give a product free from cephalin. Again the result was a negative one.

These results would indicate that there is little decomposition in the above process.

The second question to determine was the elementary composition of the lecithin obtained upon the decomposition of the cadmium salt. As we have already mentioned in the introduction, the result depends upon the character of the material which is used.

The first experiment was carried out with material which contained a considerable amount of amino nitrogen. This was decomposed in the usual manner, the lecithin obtained washed with water, and the product reduced with hydrogen. The hydro derivative was then crystallized several times from methylethyl ketone.

Sample No.	C *per cent*	H *per cent*	P *per cent*
137	63.29	10.75	3.87

The second experiment was performed on material which was practically free of amino nitrogen. The procedure was similar to that described above. Two different samples of the hydro derivatives, after several crystallizations from methylethyl ketone gave the following figures on analysis.

	Calculated:	Found:	
C	65.37	65.22	64.92
H	11.23	10.70	10.97
N	1.74	2.02	2.22
P	3.84	3.85	3.89

These results indicate, as we have mentioned above, that it is possible to obtain a pure hydrolecithin from the product of the decomposition of a pure lecithin cadmium chloride.

Preparation of Pure Hydrolecithin.—The process by which we obtained our sample of pure hydrolecithin may be outlined as follows: The egg oil, obtained upon concentrating the acetone-alcohol solution after the greater portion of the lipoids had been precipitated out, was redissolved two or three times in dry acetone, thus removing most of the lipoid material. This egg oil was then extracted with a large volume of hot alcohol, the alcoholic extract cooled to 0°, the solution decanted from the egg fat, concentrated, and the lipoid mixture (mainly lecithin with a little cephalin) precipitated with cadmium chloride. This salt was recrystallized four times from the mixture of ethyl acetate and 80 per cent ethyl alcohol. It was then free of cephalin. The salt was then decomposed according to Bergell, the lecithin washed with water, precipitated with acetone (Maclean), and reduced as

described in our first paper.[12] The analysis of the hydro body is given above.

[12] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1918, xxxiii, 111.

SOME HYDANTOIN DERIVATIVES.

By CLARENCE J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Mendel and Dakin[1] were the first to emphasize the fact that the generally accepted structural formula for allantoin contained an asymmetric carbon atom, and, because of this, should be capable of existing in two stereoisomeric forms. However, all attempts to resolve allantoin into its optically active components have failed. On the basis of this, Dakin[2] formulated a structure for allantoin which may be regarded as a tautomeric form to the one generally accepted. Dakin[3] further carried his analysis to substituted hydantoins and discovered the peculiar behavior of the optically active hydantoins towards alkali, which results in the formation of inactive forms. This reaction is remarkable in that the racemization proceeds with a much greater velocity than that observed for other substances. On the other hand, methylethylhydantoin could not be racemized.

Dakin has also made the observation that optically active amino-acids give rise to optically active hydantoins. This observation seems to be of considerable interest in connection with Dakin's theory of the structure of hydantoins. Since the active forms of the amino-acids give rise to active hydantoins, it is logical to assume that the *dl* forms of amino-acids, when first converted into hydantoins, should consist of a mixture of the *d* and the *l* forms. Further, it is logical to assume that substances consisting of such mixtures should differ in their physical properties from their tautomeric forms. It seemed of interest, therefore, to compare the physical properties—such as melting point, crystal form, refractive index—of the *dl* hydantoins and the hydantoins inactiv-

[1] Mendel, L. B., and Dakin, H. D., *J. Biol. Chem.*, 1909-10, vii, 153.
[2] Dakin, H. D., *J. Chem. Soc.*, 1915, cvii, 434.
[3] Dakin, *Am. Chem. J.*, 1910, xliv, 48.

ated by alkali. Had such differences been found, Dakin's theory would have received additional support. This consideration prompted the undertaking of the present investigation some years ago. It was found, however, that both the inactivated and the *dl* forms, in the majority of instances, did not differ in their melting points, and, roughly, in their crystal forms. At this time, it is impossible to undertake the work on the refractive indices and the work has been abandoned. It is published in its present form because of the additional data it contains on various hydantoins earlier described and because of the new hydantoins prepared during the course of this work.

	Melting point. Given. °C.	Found °C.
Hydantoin acid	155, 160	179–180
dl-Methylhydantoin	150	155–156
dl-α-Phenylureidopropionic acid	168, 170	174
dl-α-Phenylmethylhydantoin	172, 173	178
d-α-Phenylureidopropionic acid		175
d-α-Phenylmethylhydantoin		178
d-α-Naphthylureidopropionic acid		198–200
d-α-Naphthylmethylhydantoin		166
dl-α-Naphthylmethylhydantoin		179–180
Inactivated naphthylmethylhydantoin		179–180
α-Ureidobutyric acid		184
Ureidodimethylacetic acid	160	184
dl-Hydantoinacetic acid	214, 225	228–229
d-Hydantoinpropionic acid	179, 180	179–180
dl-Hydantionpropionic "	167–169, 165	179–180
Inactivated hydantoinpropionic acid	167–169	177–178
dl-*p*-Hydroxybenzylhydantoin	158–160	158–160
Inactivated benzylhydantoin	158–160	158–160
l-Hydroxybenzylhydantoin	158–160	158–160

EXPERIMENTAL.

Hydantoic Acid.—7.5 gm. of glycocoll, 8.1 gm. of potassium cyanate, and 20 cc. of water were heated on the water bath for 1 hour. The solution was filtered and concentrated hydrochloric acid added until the reaction was acid to Congo red. The acid which separated out on cooling was recrystallized from boiling water. Hydantoic acid decomposes at 179–180° (uncorrected); Diels and Heintzel[4] give the decomposition point at 155°, Weidel and Roithner,[5] as 153–154°, Griefs[6] as 160°.

[4] Diels, O., and Heintzel, H., *Ber. chem. Ges.*, 1905, xxxviii, 305.
[5] Weidel, H., and Roithner, E., *Monatsh. Chem.*, 1896, xvii, 188.
[6] Griefs, P., *Ber. chem. Ges.*, 1869, ii, 107.

Calculated for $C_3H_6O_3N_2$	N	23.73	
Found	N	23.62	23.54

Hydantoin.—Hydantoin was prepared according to the two methods of Harries and Weiss[7] and their later statement,[8] that the two products were identical, was confirmed. The melting point was found to be 218–220°, as given by Tafel and Reindl.[9]

Calculated for $C_3H_4O_2N_2$	N	28.00	
Found	N	27.80	27.82

dl-α-Methylhydantoin.—6 gm. of *dl-α*-ureidopropionic acid and 50 cc. of 10 per cent hydrochloric acid were boiled for 2 hours and the resulting solution was concentrated to a small volume. The *dl-α*-methylhydantoin which crystallized out melted at 155–156° after one recrystallization. Dakin found the melting point to be 150°.

Calculated for $C_4H_6O_2N_2$	N	24.60
Found	N	24.47

dl-α-Phenylureidopropionic Acid.—This acid was prepared by shaking 5 gm. of *dl*-alanine in 50 cc. of water containing 1.5 gm. of sodium hydroxide with 6.5 gm. of phenylisocyanate for 2 hours. The diphenylurea was filtered off and the solution acidified with dilute hydrochloric acid. The insoluble phenylureidopropionic acid separates out at once and may be recrystallized from a large volume of boiling water. *dl-α*-Phenylureidopropionic acid forms small irridescent plates which melt at 174° with decomposition. Kühn[10] gives the melting point as 170°, Paal[11] as 168°.

Calculated for $C_{10}H_{12}O_3N_2$	N	13.46
Found	N	13.47

dl-α-Phenylmethylhydantoin.—When the above ureido acid is heated with 20 per cent hydrochloric acid for 1 hour, it yields *dl-α*-phenylmethylhydantoin. Upon cooling the solution, the hydantoin crystallizes in long, silky needles which melt at 178°. Recrystallization from water did not change the melting point. Mouneyrat[12] gives the melting point at 172–173°.

Calculated for $C_{10}H_{10}O_2N_2$	N	14.74
Found	N	14.94

d-α-Phenylureidopropionic Acid.—This acid was prepared in the same way as the inactive compound. Recrystallized from boiling water, it forms small silky needles which melt at 175° with decomposition.

[7] Harries, C., and Weiss, M., *Ber. chem. Ges.*, 1900, xxxiii, 3418.
[8] Harries and Weiss, *Ann. Chem.*, 1903, cccxxvii, 355.
[9] Tafel, J., and Reindl, L., *Ber. chem. Ges.*, 1901, xxxiv, 3288.
[10] Kühn, B., *Ber. chem. Ges.*, 1884, xvii, 2884.
[11] Paal, C., *Ber. chem. Ges.*, 1894, xxvii, 976.
[12] Mouneyrat, A., *Ber. chem. Ges.*, 1900, xxxiii, 2395.

0.1000 gm. substance neutralized 9.67 cc. 0.1 N HCl.

Calculated for $C_{10}H_{12}O_2N_2$	N	13.46
Found	N	13.54

Rotation:

0.2741 gm. substance in 5.9286 gm. acetone gave a value for $[\alpha]$ of $+0.18°$.

$$[\alpha]_D^{20} = \frac{5.9286 \times 0.18°}{0.2741 \times 0.5} = +7.78°$$

d-α-Phenylmethylhydantoin.—Prepared by boiling the ureido acid with 20 per cent hydrochloric acid, the hydantoin crystallized from the hot acid solution in short pointed silky needles, which melt sharply at 178°. The melting point was not changed on recrystallization.

0.1000 gm. substance neutralized 10.37 cc. 0.1 N HCl.

Calculated for $C_{10}H_{10}O_2N$	N	14.74
Found	N	14.51

Rotation:

0.2700 gm. substance in 5.0410 gm. 0.5 N sodium hydroxide gave a value for $[\alpha]$ of $+0.06°$.

0.2031 gm. substance in 5.0988 gm. acetone gave a value for $[\alpha]$ of $-0.20°$.

$$[\alpha]_D^{20} = \frac{5.0410 \times 0.06°}{0.2700 \times 0.5} = +2.24° \text{ (sodium hydroxide).}$$

$$[\alpha]_D^{20} = \frac{5.0988 \times -0.20°}{0.2031 \times 0.5} = -10.04° \text{ (acetone).}$$

dl-α-Naphthylmethylhydantoin.—Inactive α-naphthylureidopropionic acid[13, 14] was boiled with a large excess of 20 per cent hydrochloric acid for 1 hour. A small part of the reaction product was in solution at the end and separated in long silky needles upon cooling. Most of the hydantoin, however, was in the form of an oil at the bottom of the flask and quickly solidified. Naphthylmethylhydantoin is very insoluble in boiling water but may be easily recrystallized from dilute alcohol. It melts at 179–180°.

0.1000 gm. substance neutralized 8.51 cc. 0.1 N HCl.

Calculated for $C_{14}H_{12}O_2N_2$	N	12.07
Found	N	11.91

[13] Neuberg, C., and Rosenberg, E., *Biochem. Z.*, 1907, v, 456.

[14] Gulewitsch, W., *Z. physiol. Chem.*, 1911, lxxiii, 434.

d-α-Naphthylureidopropionic Acid.—5 gm. of alanine, 10 gm. of naphthylisocyanate, and 20 cc. of 10 per cent sodium hydroxide were shaken for several hours, the reaction product was filtered, and the filtrate acidified with dilute hydrochloric acid. The acid separates at once and may be recrystallized from a large volume of boiling water or from dilute alcohol. It forms short needles, which melt at 198–200° with decomposition.

0.1000 gm. substance neutralized 7.8 cc. 0.1 N HCl.

Calculated for $C_{14}H_{14}O_3N_2$	N	11.20
Found	N	10.95

Rotation:

0.1900 gm. substance in 7.229 gm. 0.5 N sodium hydroxide gave a value for [α] of +0.05°.

$$[\alpha]_D^{20} = \frac{7.229 \times 0.05^\circ}{0.1900 \times 0.5} = +3.80^\circ$$

d-α-Naphthylmethylhydantoin.—Prepared in the same way as the inactive compound and recrystallized from boiling water or dilute alcohol, the hydantoin melts at 166°. Repeated crystallization did not raise the melting point. The inactive ureido acid crystallizes very well from 95 per cent alcohol.

0.1000 gm. substance neutralized 8.51 cc. 0.1 N HCl.

Calculated for $C_{14}H_{12}O_2N_2$	N	12.07
Found	N	11.91

Rotation:

0.2504 gm. substance in 5.2367 gm. acetone gave a value for [α] of −0.42°.

$$[\alpha]_D^{20} = \frac{5.2367 \times -0.42^\circ}{0.2504 \times 0.5} = -17.85^\circ$$

Two attempts were made to inactivate this hydantoin with normal sodium hydroxide. The first consisted in allowing the hydantoin to stand with an excess of sodium hydroxide for 2 days. At that time the rotation was zero. Because part of the compound was in the form of the sodium salt, it was warmed on the water bath before acidifying. The product which separated proved to be the inactive naphthylureidopropionic acid, decomposing at 199–200°, and yielded, when heated with 20 per cent

hydrochloric acid, the inactive hydantoin, melting at 179–180°. In the second experiment the heating on the water bath was omitted but the result was the same, the *dl*-ureido acid. Lack of material prevented the testing of a weaker alkali or of a shorter time of inactivation.

α-Ureidobutyric Acid.—10 gm. of α-aminobutyric acid, 8 gm. of potassium cyanate, and 25 cc. of water were heated 1.5 hours on a boiling water bath. The product which separated out on acidification melted, with decomposition, at 184°, both before and after crystallization from boiling water.

0.1000 gm. substance neutralized 13.0 cc. 0.1 N HCl.

Calculated for $C_5H_{10}O_3N_2$	N	19.30
Found	N	19.32

Heated 1 hour with 10 per cent hydrochloric acid, the above acid gave α-ethylhydantoin, melting at 121–122°. Koenigs and Mylo[15] found 118–120°.

Ureidodimethylacetic Acid.—10.3 gm. of α-aminoisobutyric acid, 8.1 gm. of potassium cyanate, and 25 cc. of water were heated on the water bath 1.5 hours and then acidified with concentrated hydrochloric acid. Twice recrystallized from boiling water, in which it is fairly insoluble, the acid melts at 184° with decomposition. Urech[16] gives the decomposition point as 160° and states that it is rather easily soluble in water.

Calculated for $C_5H_{10}O_3N_2$	N	19.30
Found	N	19.08

Upon heating 1.5 hours with 10 per cent hydrochloric acid, the acid forms α-dimethylhydantoin, which melts at 175–176° as given by Urech and by Heilpern.[17]

Calculated for $C_5H_8O_2N_2$	N	21.87
Found	N	21.73

p-Hydroxybenzylhydantoin.—The hydantoin from *dl*-tyrosine (Kahlbaum) and from active tyrosine were prepared according to

[15] Koenigs, E., and Mylo, B., *Ber. chem. Ges.*, 1908, xli, 4435.
[16] Urech, F., *Ann. Chem.*, 1872, clxiv, 274.
[17] Heilpern, J., *Monatsh. Chem.*, 1896, xvii, 237.

the directions of Dakin[18] and the properties as given by him confirmed.

The active compound was then inactivated by allowing a solution in N sodium hydroxide to stand about 24 hours. Upon acidification and recrystallization of the inactivated hydantoin from boiling water, it forms prisms of the same appearance as the *dl*-hydantoin, and not plates, as observed by Dakin. The melting points of the three forms, the *dl*, the *d*, and the inactivated, were the same, 158–160°, as given by Dakin.

dl-Hydantoinacetic Acid.—5 gm. of aspartic acid, 6 gm. of potassium cyanate, and 25 cc. of water were heated about 5 hours on a water bath. The resulting sirup was taken up in 10 per cent hydrochloric acid, boiled for 15 minutes, and the solution concentrated to dryness. Twice recrystallized from a little water it melts at 228–229° with decomposition. Dakin[19] gives the melting point of the inactive compound, obtained by the action of alkali upon the active acid, as 225–228°. Johnson and Guest,[20] who prepared it by the action of chloroacetic acid upon 2-thiohydantoin-4-acetic acid, give the melting point as 214–215°.

Calculated for $C_5H_6O_4N_2$	N	17.70
Found	N	17.51

dl-Hydantoinpropionic Acid.—10 gm. of *dl*-glutamic acid, 7 gm. of potassium cyanate, and 25 cc. of water were heated on a water bath for several hours until a thick sirup resulted. This was then taken up in an excess 10 per cent hydrochloric acid and boiled 20 minutes. The reaction product was evaporated to dryness in vacuum and the residue recrystallized twice from water. The acid melted at 179–180°. A second preparation showed the same melting point. Dakin[21] gives the melting point of the inactive product obtained by racemization of the active acid as 167–169°. Johnson and Guest[22] prepared the acid by desulfurizing the thiohydantoin and found the melting point to be 165°.

[18] Dakin, *Am. Chem. J.*, 1910, xliv, 55.
[19] Dakin, *Am. Chem. J.*, 1910, xliv, 58.
[20] Johnson, T. B., and Guest, H. H., *Am. Chem. J.*, 1912, xlviii, 109.
[21] Dakin, *Am. Chem. J.*, 1910, xliv, 59.
[22] Johnson and Guest, *Am. Chem. J.*, 1912, xlvii, 249.

Calculated for $C_6H_9O_4N_2$	N	16.18
Found	N	16.20

The active acid melted at 179–180° as given by Dakin.

0.2160 gm. substance in 5.2593 gm. 0.5 N sodium hydroxide gave a value for $[\alpha]$ of $-1.65°$ in a 0.5 dm. tube.

$$[\alpha]_D^{20} = \frac{5.2593 \times -1.65°}{0.2160 \times 0.5} = -80.30°$$

During the course of 6 hours this value fell from $-1.65°$ to $-1.32°$. The rate of inactivation is much more rapid in N sodium hydroxide (Dakin). The melting point of this substance was found by Dakin to be 167–169°. Crystallized from water, the melting point was found to be 177–178°.

A METHOD FOR THE DETERMINATION OF SUGAR IN NORMAL URINE.

By STANLEY R. BENEDICT and EMIL OSTERBERG.

(From the W. A. Clark Special Research Fund at the Department of Chemistry, Cornell University Medical College, with the Cooperation of W. G. Lyle, the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

(Received for publication, February 1, 1918.)

Certain observations in this laboratory convinced the present writers some time ago that a quantitative study of the substances simulating reducing carbohydrate in the urine of normal animals would be of value, and the endeavor was made to construct a satisfactory quantitative method for this purpose. The procedure described below is the outcome of nearly 3 years of work on the subject and has been tested under widely varying conditions.

We have not attempted to differentiate glucose qualitatively, but have provided for determination of "total sugar," and the sugar fermentable by yeast, by difference. The non-fermentable portion, as determined in our method, is probably a true sugar, or is at least chiefly derived from carbohydrate in metabolism. These questions will be referred to again in subsequent papers.

The procedure we have adopted is an adaptation of the colorimetric procedure of Lewis and Benedict for determination of sugar in the blood. Myers[1] has already reported an adaptation of the Lewis-Benedict method to urine, which provided for preliminary precipitation of most of the creatinine as picrate according to the suggestion of Folin. Our work with the Myers' modification has convinced us that results by this method are too high (due partly to imperfect removal of creatinine).

Indeed not infrequently the Myers method gives results at least 100 per cent too great. This is not surprising when we remember that nitrogenous compounds other than creatinine may react with picric acid in alkaline solution (allantoin, for instance

[1] Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1915-16, xiii, 178

so reacts) and that creatinine yields about five times as much color with picric acid as does an equal weight of glucose. Polyphenols are another possible source of interference.[2]

The method which we wish to present may be considered under two divisions: (1) the preliminary precipitation of interfering substances from the urine, and (2) the determination of the sugar in the filtrate. We shall discuss these processes separately before describing the technique in detail.

In the preliminary precipitation we have deemed it desirable to remove creatinine *completely*, total nitrogen as completely as possible, glycuronic acid to such an extent at least that it should not interfere appreciably in normal urines, and finally to remove the polyphenols practically completely, since these compounds also react with alkaline picric acid, although not to such an extent as does creatinine or sugar.

We have found that urines can be almost entirely freed from the substances enumerated above by a single precipitation with excess of mercuric nitrate in presence of a slight excess of sodium bicarbonate. After subsequent removal of the mercury with zinc dust the filtrates are water-clear, and contain almost no measurable nitrogen, or at least not more than 0.1 mg. per cc.[3] No creatinine can be detected in the filtrates. Folin's uric acid reagent gives only a trace of color (showing practical absence of polyphenols), and treatment of the final filtrate with basic lead

[2] It is quite surprising to note the widely diverse results obtainable for sugar in urine after precipitations aimed only to eliminate creatinine. Thus Cole (Practical Physiological Chemistry, St. Louis, 4th edition, 1914, 249) used such a procedure for qualitatively detecting abnormal quantities of glucose in urine. Later Folin (*J. Biol. Chem.*, 1915, xxii, 327) used a quite similar procedure and practically the same copper solution, for qualitatively detecting glucose in normal urine. Myers and Bailey then used the procedure for quantitative determination of small quantities of glucose in urine, and still later Hiller (*ibid.*, 1917, xxx, 125) has used Folin's precipitation method (repeated word for word) to determine only *pathological* quantities of sugar in sugar in urine—normal urine showing no sugar by this method.

[3] In most cases 20 cc. of final filtrate yield no more nitrogen by Kjeldahl than does a blank determination with the same reagents. Schöndorff (*Arch. ges. Physiol.*, 1908, cxxi, 572) employed mercury in faintly acid solution for precipitation of urines prior to titration with Fehling's solution, and reported nitrogen-free filtrates.

acetate causes no change in the sugar content, thus showing absence of any appreciable amount of glycuronic acid. Interference from creatine in the original urine is negligible unless the creatine exceeds 0.3 per cent. Such a concentration of creatine is not often encountered in urines.

For the final determination of the sugar we have materially modified the original Lewis-Benedict procedure.

A study of the reaction between picric acid and sugar in alkaline solution was undertaken with a view to increase the flexibility of the method and to eliminate the objectionable feature of boiling to dryness in a test-tube.

The results of this study may be briefly summarized as follows. Sodium hydroxide causes secondary reactions which do not go to completion and hence cannot be employed in any concentration during the reaction between picric acid and sugar.

With a constant and only moderate excess of picric acid, the depth of color given by a constant amount of sugar will increase with increase of carbonate concentration almost indefinitely. This is apparently due to products of the primary reaction, reacting with the picric acid in increasing amount with increase of carbonate. In concentrated carbonate solution the color is not directly proportional to the quantity of sugar, hence the concentration of carbonate must be kept moderate and within fixed limits.

The reaction in presence of moderate concentration of carbonate goes to completion in a moderate excess of picric acid, providing the sugar concentration is not too low. If it is desired to have the reaction proceed in a quantitative fashion where the glucose concentration may be as low as 0.5 mg. in 9 cc., this can be accomplished by suitable increase in the concentration of picric acid.

The concentration of picric acid must however, for such conditions, exceed that of a saturated aqueous solution of picric acid. This may be attained either by addition of dry picric acid, or the addition of a solution of picric acid containing considerably more than does the ordinary saturated solution. The first of these procedures is objectionable because the picric acid would have to be weighed out each time, in order to be assured of enough to cause the reaction with sugar to go to completion, and to be sure

that enough was not added to cause precipitation of sodium picrate before the final reading could be made conveniently.

We therefore endeavored to prepare a solution containing about three times the picric acid present in an ordinary saturated solution. This has been accomplished by the help of a little sodium hydroxide. Only enough hydroxide is added to convert a portion of the picric acid into sodium picrate. With the help of this solution we may start with a dilute solution of glucose in water, and, by adding some of the strong picrate solution and carbonate, obtain a final solution containing a large excess of picric acid, and one in which the reaction proceeds to quantitative completion when heated in a boiling water bath for 10 minutes.

The special reagents used in the final method for the determination of sugar in urine are:

1. Mercuric nitrate solution. It is prepared as follows: To 160 cc. of concentrated nitric acid in a beaker add in small portion, 220 gm. of mercuric oxide. When all is dissolved heat the mixture to boiling, cool, and add 60 cc. of 5 per cent sodium hydroxide solution. Make up to 1 liter and filter. Keep in a brown bottle.[4]

2. Picrate-picric acid solution. To 500 cc. of 1 per cent sodium hydroxide solution in a liter flask or stoppered cylinder add 36 gm. of picric acid, and about 400 cc. of hot water. Shake occasionally until the picric acid is dissolved, cool, and dilute to 1 liter.[5]

The final solution is fully saturated with picric acid. A slight crystallization of picric acid on standing is of no account, but if abundant crystallization occurs (due to the solution becoming too cold for some hours) it is desirable to redissolve the picric acid by warming before using.

The procedure for the sugar determination is as follows: Measure 15 or preferably 20 cc.[6] of urine into a 500 cc. beaker and

[4] The formula for this solution has been in the laboratory for a number of years. We have been unable to trace its origin.

[5] The picric acid employed should be dry and free from lumps. On account of its explosive properties, the drying of the picric acid should be carried out at a moderate temperature (60°) and the dry and cool product freed from lumps by very gentle rubbing in a mortar.

[6] If less than 15 cc. of urine are available 10 cc. may be used, but in this case the subsequent filtrations should be made on small filters and with the

add an equal volume of the mercuric nitrate solution and mix. Then add solid sodium bicarbonate in moderate quantities, with gentle shaking, until frothing ceases and the mixture reacts alkaline to litmus paper.[7] Filter at once through a dry filter into a small dry flask or beaker. To the perfectly clear filtrate add a pinch of zinc dust and 1 or 2 drops of concentrated hydrochloric acid. Shake and let stand for about 5 minutes. Filter through a small dry filter into a dry test-tube or small beaker.[8]

From 1 to 4 cc. of this final filtrate (the volume to be used depends on the sugar content of the original urine, from 0.5 to 2 mg. of sugar is the quantity desired) are measured into a large test-tube, which should be graduated to indicate 12.5 and 25 cc.[9] Where less than 4 cc. of filtrate have been used enough water is added to make exactly 4 cc. and 1 cc. of 20 per cent (anhydrous) sodium carbonate solution is added.[10] 4 cc. of the picrate-picric acid solution is now added, the tube plugged with cotton, and placed in boiling water for 10 minutes. The tube is then removed, cooled to room temperature with the help of running water, and the contents are diluted with water to the 25 cc. mark, or to 12.5 cc. if the quantity of sugar present was much below 1 mg. The

aid of suction, in order to obtain a maximum of filtrate. If fairly concentrated, the original urine may, of course, be diluted before beginning the analysis.

[7] Vigorous frothing occurs when the bicarbonate is first added—hence the use of the large beaker for this mixing. The bicarbonate may be added freely until the frothing ceases, when the mixture should be stirred or shaken *only until alkaline to litmus*. It should then be poured upon a filter at once.

[8] This filtrate should be wholly free from mercury. Until thoroughly familiar with the method it is best to test a few drops with ammonium sulfide solution. If a dark or black coloration is obtained it is necessary to mix the filtrate again with the powdered zinc, and let stand for a few minutes.

[9] Very satisfactory tubes for this purpose have been prepared for us by E. Machlett and Son of New York.

[10] Where a number of determinations are to be made it is convenient to keep on hand four solutions of sodium carbonate of different strengths, as follows: No. 1, 5 per cent; No. 2, 6.7 per cent; No. 3, 10 per cent; and No. 4, 20 per cent. Where 1 cc. of final filtrate is used add 4 cc. of Solution 1, where 2 cc. of filtrate are used add 3 cc. of No. 2, for 3 cc. of filtrate add 2 cc. of No. 3, etc., in each case giving the final solution a volume of 5 cc., and a sodium carbonate (dry) content of 0.2 gm.

colored solution is then matched (within half an hour[11]) in a colorimeter against a standard prepared by treating 1 mg. of glucose in 4 cc. of water, just as the final filtrate was treated; or against a permanent standard of picramic acid solution or potassium dichromate.

We have found that the best permanent standard is to be prepared from pure picramic acid, together with some of the picrate-picric acid solution used in the determination. This standard solution has the following composition: To 105 cc. of exactly 0.01 per cent picramic acid solution in 0.02 per cent sodium carbonate solution, add 0.5 cc. of 20 per cent sodium carbonate solution, and 15 cc. of the picrate-picric acid solution. Then dilute to 300 cc. with distilled water. Starting with pure picramic acid, the depth and quality of the color of this solution duplicate very exactly that obtained from 1 mg. of glucose dissolved in 4 cc. of water, treated as described for the final urine filtrates, and the colored solution diluted to 25 cc. The standard in the colorimeter should be set at a height of 15 mm.

The picramic acid originally prepared by the J. T. Baker Chemical Company was very satisfactory, but within the past 2 years we have seen samples of Baker's picramic acid which have yielded only about 30 per cent of the color given by an equal weight of pure picramic acid.

Where satisfactory picramic acid is not available, potassium bichromate may be employed as a standard. In our Duboscq colorimeters the dichromate solution matches the picramic acid solution standard almost exactly. In another instrument (Krüss) there is an appreciable difference in shade of color of the two solutions, but even in this instrument the dichromate standard can be employed with satisfactory results. To prepare the dichromate standard dissolve 0.536 gm. of pure potassium dichromate in water and dilute to 1 liter.

To determine the glucose or fermentable sugar in the sample of urine it is necessary to make a second determination on the sample of urine after fermentation. For fermentation we have adopted the following as a routine procedure. To 25 cc. of urine (free

[11] Where 4 cc. of final filtrate are used, the final dilution is made to only 12.5 cc.; it is necessary to make the reading promptly to avoid interference from crystallization of sodium picrate.

from preservative) in a cylinder or test-tube add 20 to 25 mg. of glucose[12] and about one-quarter cake of yeast. Mix well and allow to stand in the incubator at 35–38° for 18 to 20 hours. Decant 15 to 20 cc. of the urine and determine sugar as before fermentation. The difference between the two determinations gives the fermentable sugar.

The results obtained by this method for sugar determination in normal urine will be discussed in subsequent papers. For the present we may state that glucose added to urine is quantitatively recovered within a few thousandths of 1 per cent, and that we have considerable evidence to indicate that the method gives figures which represent very nearly the true sugar (reducing carbohydrate) content of normal urine. The procedure can be applied to diabetic urines, but most of such samples have to be diluted from 10 to 100 times before analyzing.

It is expected to apply the method to the determination of sugar in tissues in the near future. We are also studying the occurrence of polysaccharides in the urine by the help of the new method.

[12] The glucose is added as a "check" on the activity of the yeast.

A MODIFICATION OF THE LEWIS-BENEDICT METHOD FOR THE DETERMINATION OF SUGAR IN THE BLOOD.

By STANLEY R. BENEDICT.

(From the W. A. Clark Special Research Fund at the Department of Chemistry, Cornell University Medical College, with the Cooperation of W. G. Lyle, the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

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The colorimetric method for the determination of sugar in the blood which was proposed a few years ago by Lewis and Benedict has found favor at the hands of a number of investigators. In its original form the method had at least one serious drawback—the boiling to dryness in a test-tube which was necessary to complete the reaction between the sugar and picric acid.

Modifications of the method have been proposed by certain investigators to obviate this difficulty, the most useful of which have probably been the one proposed by Pearce[1] and especially that suggested by Myers and Bailey.[2] This latter modification provides for less dilution of the blood, so that the final reaction may take place in a more concentrated mixture of glucose and picric acid. If one is careful to secure full saturation of the diluted blood with picric acid the method gives quite satisfactory results for bloods high in sugar content. With normal or hypoglycemic bloods, the results are not so good, and duplicates on such bloods will frequently vary quite considerably. Nevertheless, the method has unquestionably been of distinct service in the hands of many investigators.

The method to be described below retains the same dilution of the blood as that employed by Lewis and Benedict,[3] eliminates

[1] Pearce, R. G., *J. Biol. Chem.*, 1915, xxii, 525.

[2] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

[3] The writer believes that both theoretically and practically a dilution of 1 to 12.5 is preferable to the dilution of 1 to 5 as adopted by Myers and Bailey.

the necessity of saturating with dry picric acid, and has a very simple technique for the final reaction.

The procedure is as follows: 2 cc. of blood are drawn into an Ostwald pipette, containing a little powdered potassium oxalate, and discharged into a 25 cc. graduated flask, or into a large test-tube graduated at 12.5 and at 25 cc. The pipette is twice rinsed out with distilled water, these washings being added to the blood. After a minute or two the blood is practically completely laked. A solution of sodium picrate and picric acid,[4] is added to the 25 cc. mark (using a few drops of alcohol to dispel foam if necessary) and the mixture thoroughly shaken. After a minute or two (or longer) the mixture is poured upon a dry filter, and the clear filtrate collected in a dry beaker. Exactly 8 cc. of the filtrate are measured into a large test-tube bearing graduations at the 12.5 cc. and 25 cc. mark, and 1 cc. of 20 per cent (anhydrous) sodium carbonate solution is added. The tube is plugged with cotton and immersed in boiling water for 10 minutes.[5] It is then removed, and the contents are cooled under running water and diluted to 12.5 cc. or to 25 cc. depending on the depth of color. At any time within half an hour the colored solution is compared in a colorimeter with a suitable standard solution,[6] the standard being set at a height of 15 mm.

The standard solution may be simultaneously prepared from pure glucose by treating 0.64 mg. of glucose in 4 cc. of water with 4 cc. of the picrate-picric acid solution and 1 cc. of the carbonate, and heating for 10 minutes in boiling water and then diluting to 12.5 cc.

A permanent standard solution may be prepared from picramic acid or from potassium dichromate. The latter standard does not match the unknown with absolute exactness, but can be

[4] This solution is the same as that described in the preceding paper. To prepare it, place 36 gm. of dry powdered picric acid in a liter flask or stoppered cylinder, add 500 cc. of 1 per cent sodium hydroxide solution, and 400 cc. of hot water. Shake occasionally until dissolved. Cool and dilute to 1 liter.

[5] Longer heating up to half an hour makes no change in the color.

[6] Occasionally the final filtrates in this or other picric acid methods develop a little turbidity during heating. Unless such turbidity is fairly marked it is of no account. When desired, the final colored solution may be filtered through a small folded filter into the colorimeter cup.

employed with satisfactory results when pure picramic is not obtainable.

The picramic acid standard is best prepared from a stock solution containing 100 mg. of picramic acid and 200 mg. of sodium carbonate per liter. 126 cc. of this solution are treated with 1 cc. of the 20 per cent carbonate solution and 15 cc. of the picrate-picric acid solution, and diluted to 300 cc. with distilled water. This solution matches exactly the color from 0.64 mg. of glucose, when treated as in the above method and diluted to 12.5 cc.

The standard prepared from potassium dichromate contains 800 mg. of pure potassium dichromate in a liter of water.

Results obtained by the method described above duplicate very closely those obtained by the original Lewis-Benedict method for the same samples of blood. The use of the three times saturated picric acid solution provides an abundance of picric acid in the final filtrate so that the reaction goes to completion without further concentration.

If desired, 1 cc. of blood may be used instead of 2, making the initial dilution to 12.5 cc. instead of to 25 cc. In this case the test-tube graduated at 12.5 cc. and at 25 cc. is convenient for the dilution. Where 1 cc. of blood is employed there is not sufficient filtrate for duplicates, but after one is used to the method, duplicates are scarcely necessary.

The calculation is $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \div 10 =$ per cent of sugar in the original blood. Where the final dilution is made to 25 cc. instead of 12.5 cc. the final figure is of course multiplied by two.

For clinical purposes Epstein[7] has modified the Lewis-Benedict method so as to make it applicable to the determination of sugar in 0.2 cc. of blood. Epstein published this modification while using the Lewis-Benedict method through the courtesy of a private communication to the Mt. Sinai laboratory. His paper appeared prior to the final paper of Lewis and Benedict, and was published without consulting Lewis and Benedict in any way or asking whether they had a similar clinical application in mind. The present writer wishes therefore to reserve, for a reasonable

[7] Epstein, A. A., *J. Am. Med. Assn.*, 1914, lxiii, 1667.

time, application of the method described in the present paper to quantities of blood smaller than 1 cc.

In conclusion it may be of interest to answer, at least partially, a verbal criticism of the Lewis-Benedict method which has come occasionally to the attention of the present writer. This criticism has been substantially that the picric acid method yields high results for blood, owing to the production of color by non-carbohydrate substances—most notably creatine and creatinine.

Evidence against the picric acid method has been lacking, but the criticism has recurred at not infrequent intervals. It has been widely recognized that the method gives results for human blood essentially duplicating those obtained by standard methods, so that the criticism has been chiefly limited to dog's blood, concerning the sugar content of which there has of late years been considerable discussion.

The writer has therefore determined the sugar content of several samples of dog's blood by (*a*) the method described in the present paper and (*b*) by application of the procedure described for urine in the preceding paper. In the latter case the blood was first coagulated with five volumes of 0.01 N acetic acid by boiling, an aliquot of the filtrate concentrated by boiling to a suitable volume, and this solution treated exactly as described for urine in the preceding paper. On the five different samples of blood tested, the mercuric nitrate precipitation method gave results only 2 to 4 per cent lower than the direct picric acid method, as indicated in the following table.

Dog's Blood.

Sample No	Direct picric acid method.	Preliminary precipitation with $Hg(NO_3)_2$ and $NaCHO_2$.
	per cent	*per cent*
1	0.093	0.091
2	0.12	0.097
3	0.090	0.088
4	0.095	0.097
5	0.14	0.11

A control by fermentation showed that slightly over 90 per cent of the sugar, as determined by the direct picric acid method, is fermented by yeast.

We contemplate a study of the sugar content of pathological human bloods along the lines above indicated. We are inclined to believe that in some instances, particularly in advanced nephritis, interfering substances may be of enough significance to warrant recourse to preliminary precipitation with mercuric nitrate and bicarbonate.

STUDIES IN CARBOHYDRATE METABOLISM.

I. A PRELIMINARY REPORT ON THE SUGAR ELIMINATION IN THE URINE OF THE NORMAL DOG.

By STANLEY R. BENEDICT and EMIL OSTERBERG.

With the Technical Assistance of Cecil Dudley.

(From the W. A. Clark Special Research Fund at the Department of Chemistry, Cornell University Medical College, with the Cooperation of W. G. Lyle, the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

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An accurate study of the urinary sugar elimination of normal animals has hitherto been practically impossible, owing to the lack of suitable technique. We have applied the method for urinary sugar determination described in one of the preceding papers to the urine of the dog, and the present paper presents a summary of some of our findings upon one animal. Several other animals have been studied in sufficient detail to warrant the conclusion that the results presented in the present paper hold essentially for the species.

As is noted in a preceding paper,[1] our method provides for determination of both fermentable and unfermentable sugar. In the present paper we are reporting results for total sugar in the urine of the dog. In subsequent communications we shall discuss the relationship of fermentable to unfermentable sugar in the urine of the dog, and shall show that it is possible to vary either or both of these two constituents of the urine by suitable administration of carbohydrate.

The present work has been planned to determine: (*a*) the total sugar excretion upon various diets and during starvation, and (*b*) the sugar output in relation to the time of food ingestion and the urinary volume. This latter point is of especial significance because through such a study we may find out whether urinary sugar excretion is simply due to the fact that the kidney is an only partially efficient filter, so that a constant percentage of sugar

[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

passes through with the other urinary constituents, or whether urinary sugar, even in normal animals, is at least partially a measure of carbohydrate tolerance.

In our plan to follow the urinary sugar in frequent periods throughout the entire 24 hours we have been greatly helped by a machine constructed by one of us (D.) which provides for collection of each sample separately at the time of urination, while simultaneously recording the exact time of urination upon a revolving drum. We have therefore not catheterized during these experiments. The irregular hours of urine collection recorded in the tables represent the hours of spontaneous urination. Since the machine may prove of service to others, we shall describe it in detail in a subsequent communication.

Table I represents our findings with a bitch of a weight of about 18 kilos, upon a diet such as we commonly feed laboratory animals and which is rather high in carbohydrate content.

An inspection of Table I will show that on a constant diet the total urinary sugar output for the 24 hour period is relatively constant. The quantity eliminated on this diet averages about 17 mg. per hour for the 24 hours, with a maximum variation for different days of 15.6 mg. to 16.8 mg. per hour, for the 24 hour period. It will be noted that the sugar output is *totally independent of urinary volume*. The *percentage* of sugar in the urine varies enormously, as illustrated by the maximum, 0.36 per cent (one sample on April 8), and the minimum, 0.021 per cent (one sample on April 15).

In spite of the fact that the diet contained very little free sugar (only about 4 gm. of lactose in the milk) it will be noted from Table I that *food ingestion is the controlling factor in the sugar elimination*. An inspection of the output of sugar per hour for the various periods after food ingestion shows a very definite course for the sugar elimination.

This fact is perhaps most clearly brought out on April 15 (Table I). During this day the dog urinated eleven times during the 24 hours, so that the sugar elimination can be followed in somewhat more detail than during the other days. For the first 2 hours after food ingestion the sugar elimination is 13.5 mg. per hour. During the following hour this rate is approximately doubled to 26 mg. per hour. For the next hour and a half the rate of elimina-

TABLE I.

Total Urinary Sugar Output on Ordinary (Carbohydrate) Diet. Bitch of About 18 Kilos Weight.

Diet.	*gm.*
Cracker meal	135
Meat	50
Evaporated milk	50
Dog biscuit	40
Lard	20
Bone ash	20

Date.	Volume.	Reaction.	Total sugar.		
1917	*cc.*		*per cent*	*mg.*	*mg. per hr.*
Apr. 13					
Fed. 10.00 a.m.					
9.55 a.m.– 3.00 p.m.	85.0	Alkaline.	0.124	105.3	21.1
3.00 p.m.– 5.15 "	73.0	"	0.102	74.5	33.1
5.15 " – 7.45 "	128.0	Acid.	0.051	65.3	26.1
7.45 " –11.45 "	81.0	"	0.10	81.0	20.2
11.45 " – 2.30 a.m.	80.0	"	0.031	24.8	9.0
2.30 a.m.– 5.45 "	80.0	"	0.038	30.4	9.4
5.45 " – 8.45 "	80.0	"	0.027	21.6	7.2
	607.0		0.0664	402.9	17.7
Apr. 14					
Fed. 9.20 a.m.					
8.45 a.m.–10.00 a.m.	9.0	Slightly alkaline.	0.083	7.5	6.0
10.00 " –12.00 "	23.0	Alkaline.	0.170	39.4	19.7
12.00 " – 3.00 p.m.	48.0	"	0.179	86.0	28.7
3.00 p.m.–10.55 a.m.	475.0	Acid.	0.058	275.5	13.8
	555.0		0.0736	408.4	15.6
Apr. 15					
Fed. 11.00 a.m.					
10.55 a.m.– 1.00 p.m.	16.0	Alkaline.	0.168	26.9	13.5
1.00 p.m.– 2.00 "	12.0	"	0.217	26.0	26.0
2.00 " – 3.30 "	51.0	"	0.08	40.8	27.2
3.30 " – 4.00 "	59.0	"	0.035	20.7	41.4
4.00 " – 5.30 "	38.0	"	0.103	39.1	26.0
5.30 " – 8.20 "	94.0	Slightly alkaline.	0.098	92.1	30.7
8.20 " –10.10 "	31.0	Acid.	0.113	35.0	20.0
10.10 " –12.10 a.m.	28.0	"	0.10	28.0	14.0
12.10 a.m.– 2.15 "	76.0	"	0.027	20.5	10.3
2.15 " – 6.00 "	85.0	"	0.021	17.8	4.7
6.00 " – 9.30 "	66.0	"	0.030	19.8	5.65
	556.0		0.066	366.7	16.3

TABLE I—*Concluded.*

Date.	Volume.	Reaction.	Total sugar.		
1917	*cc.*		*per cent*	*mg.*	*mg. per hr.*
Apr. 16					
Fed, 10.30 a.m.					
9.30 a.m.– 2.15 p.m.....	42.0	Alkaline.	0.152	63.8	13.4
2.15 p.m.– 5.15 "	63.0	"	0.160	100.8	33.6
5.16 " – 6.25 "	19.0	Slightly alkaline.	0.155	29.5	23.6
6.25 " –10.55 "	64.0	Acid.	0.108	69.1	15.4
10.55 " – 2.10 a.m.....	55.0	"	0.088	48.4	14.9
2.10 a.m.– 7.50 "	150.0	"	0.035	52.5	9.1
7.50 " – 9.30 "	17.0	"	0.071	12.1	8.1
	410.0		0.092	376.2	15.7
Apr. 17					
Fed, 9.40 a.m.					
9.30 a.m.–11.30 p.m.....	236.0	Slightly alkaline.	0.120	283.2	20.2
11.30 p.m.– 9.15 a.m.....	224.0	Acid.	0.048	107.5	11.0
	460.0		0.085	390.7	16.8
Apr. 18					
Fed, 10.10 a.m.					
9.15 a.m.– 9.50 p.m.....	358.0	Alkaline.	0.077	275.7	22.1
9.50 p.m.– 4.40 a.m.....	116.0	Acid.	0.059	68.5	9.8
4.40 a.m.– 6.40 "	90.0	"	0.028	25.2	12.6
6.40 " –10.00 "	39.0	"	0.065	25.4	7.8
	603.0		0.0655	394.8	16.0

tion is 27.2 mg. per hour, followed during the next half hour (4.5 to 5 hours after feeding) by an increase to the maximum of the day—41.4 mg. per hour. From this maximum there is a slow and almost continuous drop in the sugar output (broken once by a slight rise from 5.30 to 8.20 p.m.) to the minimum of 4.7 mg. per hour, which is reached about 15 hours after food ingestion. The experiments on other days with the same diet record the same general course for the sugar elimination. It may be noted here that during the periods of the minimum sugar elimination, as illustrated on April 15 between 12.10 and 9.30 a.m., glucose

TABLE II.

Same Animal on Protein (Carbohydrate-Poor) Diet.

Diet.	*gm.*
Meat	300
Lard	40
Cracker meal	50
Bone ash	20

Date.	Volume.	Reaction.	Total sugar.		
1917	*cc.*		*per cent*	*mg.*	*mg. per hr.*
Mar. 27					
Fed, 11.15 a.m.					
11.10 a.m.– 3.40 p.m.	86.0	Alkaline.	0.065	55.9	12.4
3.40 p.m.– 6.20 "	88.0	Slightly alkaline.	0.078	68.8	25.0
6.20 " – 8.15 "	36.0	Acid.	0.065	23.4	11.7
8.15 " – 3.15 a.m.	168.0	"	0.034	57.1	8.2
3.15 a.m.– 9.00 "	126.0	"	0.040	50.4	8.8
9.00 " –11.35 "	16.0	"	0.110	17.6	7.0
	520.0		0.0525	273.2	11.15
Mar. 28					
Fed, 11.35 a.m.					
11.35 a.m.– 6.35 p.m.	100.0	Alkaline.	0.100	100.0	14.3
6.35 p.m.– 8.50 "	40.0	Acid.	0.029	11.6	5.2
8.50 " –12.45 a.m.	62.0	"	0.075	46.5	11.6
12.45 a.m.– 9.15 "	150.0	"	0.058	87.0	10.1
	352.0		0.070	245.1	9.8
Mar. 29					
Fed, 9.15 a.m.					
9.15 a.m.– 2.50 p.m.	80.0	Alkaline.	0.134	107.2	19.5
2.50 p.m.– 7.25 "	81.0	Acid.	0.045	36.4	8.1
7.25 " –11.35 "	53.0	"	0.088	46.6	11.0
11.35 " – 4.00 a.m.	48.0	"	0.084	40.3	9.0
4.00 a.m.–10.15 "	65.0	"	0.096	62.4	10.0
	327.0		0.089	292.9	11.7
Mar. 30					
Fed, 10.15 a.m.					
10.15 a.m.– 9.00 p.m.	195.0	Acid.	0.083	162.0	15.1
9.00 p.m.– 8.00 a.m.	130.0	"	0.090	117.0	10.6
	325.0		0.086	279.0	12.8

TABLE II—*Concluded.*

Date.	Volume.	Reaction.	Total sugar.		
1917	*cc.*		*per cent*	*mg.*	*mg. per hr.*
Mar. 31					
Fed, 10.10 a.m.					
8.00 a.m.– 2.20 p.m.....	90.0	Acid.	0.112	100.8	16.1
2.20 p.m.– 8.20 "	150.0	"	0.05	75.0	12.5
8.20 " – 6.45 a.m.....	145.0	"	0.056	81.2	7.7
6.45 a.m.–11.00 "	65.0	"	0.091	59.2	13.9
	450.0		0.0703	316.2	11.7

frequently completely disappears from the urine, as indicated by no change in the sugar content of the urine after fermentation with yeast. This point will be discussed in more detail in a subsequent paper.

The high variation in the *percentage* of sugar in the urine is of interest as indicating absence of influence of urinary volume upon the absolute amount of sugar eliminated.

In Table II are recorded experiments with the same animal upon a diet high in protein and low in carbohydrate. This diet contained no free sugar.

It will be noted that the total sugar elimination on the meat diet is appreciably less than on the diet containing more starch. On this latter diet the dog had an average sugar elimination of about 17 mg. per hour, while on the low carbohydrate diet this figure falls to about 11 mg. per hour. The ingestion of the meat diet is followed by a rise in the urinary sugar as in the case of the carbohydrate diet, but the rise is not to so high a figure, nor does it extend over so long a period after the ingestion of the food.

In Table III the results for 4 days of fasting are reported. For these days the sugar elimination is nearly constant from hour to hour and averages about 8 mg. per hour. This figure is appreciably lower than that obtained with the meat diet, and almost as low as was obtained for any of the periods representing from 16 to 24 hours after food ingestion on either the meat or the carbohydrate diet. Thus the *total sugar* elimination of the well fed normal dog falls to a true fasting level during approximately the last 8 or 10 hours of the 24. We have some data, however, which show that the ratio of fermentable sugar (glucose) to the total

sugar varies markedly during more prolonged fasting from the ratio which obtains during the fasting hours of the animal fed once a day.

In concluding it seems desirable to call attention in a general way, to the reaction (to litmus) of the urine samples as reported in our experiments. Alkaline reactions are reported so frequently that the reader may be suspicious that we were dealing with an

TABLE III.

Same Animal Fasting.

Date.	Volume.	Reaction.	Total sugar.		
1917	*cc.*		*per cent*	*mg.*	*mg. per hr.*
Apr. 2					
11.00 a.m.–10.30 p.m.....	72.0	Acid.	0.128	92.2	8.0
10.30 p.m.–10.30 a.m.....	71.0	"	0.126	89.5	7.5
	143.0		0.127	181.7	7.7
Apr. 3					
10.30 a.m.– 9.00 a.m.....	72.0	Acid.	0.286	206.0	9.1
Apr. 4					
9.00 a.m.–10.45 p.m.....	66.0	Slightly alkaline.	0.193	127.3	9.3
10.45 p.m.– 9.00 a.m.....	50.0	Acid.	0.148	74.0	7.2
	116.0		0.1735	201.3	8.4
Apr. 5					
9.00 a.m.–11.45 a.m.....	48.0	Slightly alkaline.	0.051	24.5	8.9
11.45 " –10.20 "	80.0	Acid.	0.21	168.0	7.5
	128.0		0.150	192.5	7.6

animal having an infected bladder. We may state definitely that this was not the case. The animal was not catheterized at any time. All mixed 24 hour samples were strongly acid. The chief "alkaline tide" invariably follows food ingestion, and during such a period samples $\frac{1}{2}$ hour apart were just as definitely alkaline as those obtained at longer intervals following the food ingestion. The night samples, which frequently remained in the bladder for

many hours were strongly acid. Other animals we have studied have shown the same general "alkaline tide" encountered in this one. We are probably simply recording in some detail the commonly recognized fact that urines tend to become less acid for a period during digestion.

A comparison of the reaction of the urine with the quantity of sugar eliminated shows that the alkaline urines contain the larger quantities of sugar. The transition to acid reaction is practically invariably accompanied by a sharp fall in the rate of sugar elimination. Whether these facts are causally interrelated cannot be answered at present. It may be noted however, that such a finding might be in accord with the view of a number of recent investigators who maintain that there is a definite relationship between hydrogen ion concentration of the blood and sugar utilization by the organism.

This question, together with various other phases of the problem of carbohydrate utilization in the dog under normal and certain pathological conditions will be reported upon in further communications. In the near future we shall report upon the alleged "glucose tolerance" in the dog and upon the changes of the urinary sugar forms following the introduction of carbohydrate by various means.

STUDIES IN CARBOHYDRATE METABOLISM.

II. A STUDY OF THE URINARY SUGAR EXCRETION IN TWO NORMAL MEN.

By STANLEY R. BENEDICT, EMIL OSTERBERG, AND ISAAC NEUWIRTH.

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Few subjects in biological chemistry have received as much attention, with as little definite result, as the question of the sugar excretion in normal urine. Extended investigations, such as that of Baisch[1] have indeed shown that normal human urine habitually contains carbohydrate, a portion of which is probably dextrose. Baisch also detected a second sugar which was nonfermentable with yeast, and which yielded an osazone melting at about 153°. He regarded this sugar as probably identical with isomaltose. Pavy and Siau[2] reported similar findings for urine, blood, and tissue and concluded positively that glucose is not the *only* reducing sugar present in the body. Attempts to study the normal sugar elimination and the factors influencing it have not been wanting, but the results have been inconclusive, owing chiefly to lack of suitable analytical technique. Moritz[3] in 1890 reported a detailed study of the copper-reducing substances in normal urine, but concluded little more than that dextrose is probably present.

Breul[4] attempted a quantitative study of normal sugar excretion and concluded that urine ordinarily contains 0.027 to 0.178 per cent of sugar, amounting to an excretion of 0.3 to 1.9 gm. per

[1] Baisch, K., *Z. physiol. Chem.*, 1895, xx, 248.

[2] Pavy, F. W., and Siau, R. L., *J. Physiol.*, 1900–01, xxvi, 282.

[3] Moritz, F., *Deutsch. Arch. klin. Med.*, 1890, xlvi, 217.

[4] Breul, L., *Arch. exp. Path. u. Pharm.*, 1898, xl, 1.

day for an ordinary adult man. He further concluded that feeding with carbohydrate does not increase the normal sugar elimination. Schöndorff,[5] making use of a much better technique, found that the normal dextrose content of urine averages from 0.01 to 0.02 per cent and reported that ingestion of high carbohydrate diets would increase the sugar content of normal urines very markedly—up to 0.1 per cent. More recently Oppler,[6] working with urines precipitated with lead and phosphotungstic acid, concludes that urine may contain an optically inactive, fermentable sugar up to 0.04 per cent, but that true dextrose cannot be present up to more than 0.01 per cent.

Thus the information at present available is not only vague in regard to the nature of the urinary sugars, but is equally or more vague concerning the factors which influence the excretion of sugar in normal urine. Recent procedures, such as that suggested by Folin,[7] to demonstrate dextrose in normal urine serve to call attention again to the fact that urine contains traces of reducing substance, but afford no positive data beyond this point. Indeed the knowledge of normal sugar excretion has not been advanced by such procedures beyond the evidence offered by Crismer[8] years ago. Crismer reported that safranin in alkaline solution is reduced by normal urines, but is unaffected by any known constituent of urine other than dextrose. The use of Crismer's solution (0.1 per cent safranin in water) stands today as the simplest method of demonstrating the *probable* presence of dextrose in normal urine, and is theoretically quite as accurate as any of the more elaborate procedures so far proposed.

Our lack of knowledge of the factors involved in normal sugar excretion is perhaps nowhere more emphasized than in the textbooks on diabetes. Even the best of these dismiss the question of sugar in normal urine with a few lines of vague and uncertain statements.[9] Yet the problem of diabetes must be intimately bound up with the facts of normal sugar excretion. Perhaps one

[5] Schöndorff, B., *Arch. ges. Physiol.*, 1907, cxxi, 572.

[6] Oppler, B., *Z. physiol. Chem.*, 1911, lxxv, 71.

[7] Folin, O., *J. Biol. Chem.*, 1915, xxii, 327.

[8] Crismer, L., *Ann. Soc. méd-chir. de Liége*, 1888, October.

[9] Joslin, E. P., Treatment of Diabetes Mellitus, Philadelphia and New York, 2nd edition, 1917.

of the chief difficulties in solving the problem of diabetes lies in the fact that diabetes represents primarily the absence, or minimal activity, of a function which the investigator is trying to study. In line with this view it is worthy of note that Allen made his progress in the treatment of diabetes after studies on animals in which there was (through operative technique) minimal impairment of the carbohydrate function. Allen's main conclusions from his studies on partially depancreatized dogs are not only corroborated by the findings on the normal dog reported in the preceding paper, but it is interesting to note that his main conclusions concerning the therapeusis of diabetes might have been reached originally wholly from the normal animal.

In the present paper we are presenting a rather detailed report of the quantitative urinary sugar findings for two normal men. Not all of the experimental data obtained have been included, but we have tried to report in sufficient detail so that there may be no question as to the basis for the conclusions drawn.

The general plan of the work has been as follows: The individuals selected were both thoroughly familiar with the routine of metabolism experiments and with the methods of study involved in the present work. Neither subject has a direct diabetic history in the family. In age and type the subjects vary widely.

E. O., age 51 years, weight 86 kilos, has been an instructor in the laboratory for many years. His urine has been frequently qualitatively examined for sugar, during this period, with negative results.

I. N., age 22 years, weight about 57 kilos, has been a graduate student in the laboratory for 2 years. His urine has given negative qualitative tests for sugar with the Benedict reagent and there is no history of diabetes in his family. He is of muscular build, active, and a light eater.

The method for sugar determination in the urine employed throughout the present work was a slight modification of the one described in the first paper of the present series. Where sugar is present up to 0.025 per cent in the original urine this method will determine it to an accuracy of about 0.004 per cent.[10]

[10] For a discussion of the adequacy of this method for the present study, the reader is referred to the first of the preceding papers (Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195).

The work at present reported covers the following specific points for the two individuals. (1) The total sugar output for 24 hours on various diets (normal, high carbohydrate, and low carbohydrate). Aside from its fundamental importance, an answer to this question for a number of individuals should afford valuable data for clinicians attempting to diagnose borderline cases of diabetes; *i.e.*, those cases in which an individual sample of urine may show a questionable reaction with any or all of the qualitative copper tests. An accurate determination on the 24 hour sample of such an individual will, when sufficient normal data are available, afford a definite basis for diagnosis which can be obtained in no other way. (2) The question has been studied as to the excretion of sugar in relation to the ingestion of food. (3) The tolerance to glucose and to cane sugar on an empty stomach has been studied. Since our method provides for the determination of smaller quantities of sugar than has hitherto been possible and since we use a new criterion to determine tolerance, it seemed probable that previous conclusions on this point might be somewhat erroneous. Our results have shown that this is the case. (4) The effects of addition of varying amounts of glucose and of cane sugar to a weighed diet were studied. Our results on the relationship of sugar elimination to food ingestion led to this study. (5) Throughout the work we have carefully determined both the fermentable sugar (dextrose?) and the unfermentable sugar in all samples of urine, and have recorded both of these in mg. and in per cent of the total sugar.

When we began our work on sugar in urine we had the common impression that the only sugar of any account in urine is glucose, and we believed our method at fault when we could not obtain zero readings after fermentation. But when we found that our final filtrates were practically nitrogen-free, and that the non-fermentable portion increased with increase of carbohydrate in the diet, as did the fermentable portion, we were led to change this opinion. Observations, that increase of protein in the diet led to no increase of the non-fermentable sugar, further strengthened the view that the non-fermentable substance is derived from carbohydrate and should be classed as a sugar. Certain observations on the dog, which we will report later, seem to prove the correctness of this latter position. We expect to be

able to isolate the non-fermentable substance from the urine of the dog.

At present we regard the occurrence and relationship of the unfermentable carbohydrate as of the greatest significance. Indeed, as will be noted in several of our experiments, the first detectable effect on the urine of sugar given in small quantities is a changed relationship of the fermentable and non-fermentable sugars as determined in our method.

EXPERIMENTAL.

Table I presents the urinary sugar findings for E. O. upon an ordinary mixed diet. The urines were collected in 12 hour periods. In spite of the fact that the diet was not weighed, the total sugar excreted is quite constant for the 5 days, ranging between 0.903 and 1.161 gm. for the 24 hour period. The average total sugar elimination is about 1 gm. per day.

The quantity of sugar excreted is quite independent of volume of the urine, the percentage of sugar in the urine ranging between 0.067 and 0.124 per cent. The greater quantity of the sugar is eliminated during the night period. Subsequent tables will show that this is due to the fact that the chief meal of the day was eaten at 7 p.m. The fermentable sugar (dextrose?) ranges between 356 and 571 mg. The unfermentable sugar varies between 367 and 660 mg., and between 40 and 65 per cent of the total. These variations are probably due to differences in the diet, since they are not nearly so apparent upon the second 2 days on a weighed diet (Table VIII). The percentage of the unfermentable sugar is invariably higher for the day sample, where the total sugar elimination is less.

Table II records results for the same individual upon a diet low in carbohydrate and high in protein. Excepting the first day of this diet (to exclude any after effect of preceding diet) we note that the total sugar eliminated is remarkably constant, ranging between 0.791 and 0.723 gm. with an average of about 0.75 gm., as contrasted with an average of about 1 gm. on the ordinary diet. Both fermentable and unfermentable sugar are appreciably lower than on the ordinary diet. The urinary volumes are considerably greater than on the preceding diet, so that the percentage of sugar in the urine is very much lower than on the ordinary diet. This

TABLE I.

E. O.—Fermentable and non-fermentable sugar in urine on ordinary (unweighed) mixed diet. Urine collected in 12 hour periods. Details of diet follow the figures for each day.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	cc.	per cent	mg.	mg.	per cent	mg.	per cent
June 21							
7 a.m.–7 p.m.	400	0.098	392.0	128.0	0.066	264.0	67.3
7 p.m.–7 a.m.	600	0.104	624.0	228.0	0.066	396.0	63.5
7 a.m.–7 a.m.	1,000	0.1016	1,016.0	356.0	0.066	660.0	65.0

Breakfast: Cornbread, milk, butter, sugar, coffee.....7.30 a.m.
Lunch: Bread, butter, eggs, cheese.....1.30 p.m.
Dinner: Steak, vegetables, bread, butter, cake, tea, milk, sugar.....7.30 "

June 22							
7 a.m.–7 p.m.	420	0.124	521.4	206.4	0.075	315.0	60.4
7 p.m.–7 a.m.	640	0.100	640.0	364.7	0.043	275.3	43.0
7 a.m.–7 a.m.	1,060	0.1095	1,161.4	571.1	0.055	590.3	50.8

Breakfast: Force, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Bread, butter, eggs, cheese.....1.00 p.m.
Dinner: Clam chowder, weakfish, bread, butter, potatoes, coffee, milk, sugar, starch pudding, cake.....7.30 "

June 23							
7 a.m.–7 p.m.	480	0.082	393.6	187.2	0.043	206.4	52.4
7 p.m.–7 a.m.	800	0.067	536.0	288.0	0.031	248.0	46.3
7 a.m.–7 a.m.	1,280	0.073	929.6	475.2	0.035	454.4	48.9

Breakfast: Force, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Cold meat, bread, butter.....1.30 p.m.
Dinner: Bread, butter, canned salmon, glass of milk, western egg sandwich.....7.30 "

June 24							
7 a.m.–7 p.m.	510	0.073	372.4	219.4	0.030	153.0	41.1
7 p.m.–7 a.m.	690	0.076	531.3	317.3	0.031	214.0	40.3
7 a.m.–7 a.m.	1,200	0.0753	903.7	536.7	0.030	367.0	40.6

Breakfast: Eggs, lamb chops, bread, butter, milk, sugar, coffee.....7.00 a.m.
Lunch: Pot roast, potatoes, onions, bread, butter, tea, milk, sugar.....1.30 p.m.
Dinner: Bread, butter, cheese, peaches, cream, coffee, sugar.....7.30 "

TABLE I—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
June 25							
7 a.m.–7 p.m.....	450	0.079	355.5	135.0	0.049	220.5	62.0
7 p.m.–7 a.m.....	560	0.110	616.0	319.2	0.053	296.8	48.2
7 a.m.–7 a.m.....	1,010	0.096	971.5	454.2	0.051	517.3	53.2

Breakfast: Orange, force, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Bread, butter, meat, cheese.....1.00 p.m.
Dinner: Veal chops, potatoes, tomatoes, onions, radishes, bread, butter, milk, sugar, tea.....7.30 "

point illustrates again the absence of any direct relationship between quantity of urine and of sugar. The percentage of non-fermentable sugar averages higher on the protein than on the ordinary diet.

Table III presents detailed results upon a high carbohydrate diet. The urine was collected and analyzed in 2 hour periods from 5 a.m. to 11 p.m. The total sugar output on the diet high in carbohydrate is about 50 per cent greater than on the ordinary diet, averaging nearly 1.5 gm. per day. This figure is almost double that on the high protein diet. The absolute amount of non-fermentable sugar is also markedly higher than on either of the preceding diets, amounting to about 0.6 gm. per day. Though increased in absolute amount, the *percentage* of the non-fermentable sugar is lower than on the ordinary or high protein diets, averaging only slightly over 40 per cent of the total sugar. A comparison of the absolute amounts of non-fermentable sugar eliminated upon the three diets shows unmistakably the origin of this substance in carbohydrate.

The course of the sugar excretion for the 2 hour periods is of interest. From 5 to 7 a.m. each day the sugar eliminated is nearly at a minimum, while the percentage of non-fermentable sugar is at practically its maximal figure. For the 2 hours following breakfast the total sugar eliminated is about 300 per cent higher than for the two previous fasting hours. The extra

TABLE II.

E. O.—Fermentable and non-fermentable sugar in urine on carbohydrate-poor (high protein) diet. Urine collected in 12 hour periods. Details of the diet follow the figures for each day.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 8							
7 a.m.–7 p.m.....	510	0.093	474.3	209.1	0.052	265.2	55.9
7 p.m.–7 a.m.....	710	0.054	383.4	92.3	0.041	291.1	75.9
7 a.m.–7 a.m.....	1,220	0.070	857.7	301.4	0.045	556.3	64.8

Breakfast: Two eggs, frankfurters, pint of milk, bread, butter.
Lunch: Roast chicken, peas, bread, butter, pint of milk, crackers, cheese.
Dinner: Cold ham, chicken, onions, bread, butter, ½ pint milk.

July 9							
7 a.m.–7 p.m.....	584	0.059	344.5	134.5	0.036	210.0	61.0
7 p.m.–7 a.m.....	910	0.045	410.0	182.5	0.025	227.5	55.5
7 a.m.–7 a.m.....	1,494	0.0505	754.5	317.0	0.029	437.5	58.0

Breakfast: Two eggs, pint of milk, bread, butter.
Lunch: Dried beef, bread, butter.
Dinner: Steak, pint of milk, bread, butter.

July 10							
7 a.m.–7 p.m.....	705	0.052	366.6	190.3	0.025	176.3	48.1
7 p.m.–7 a.m.....	850	0.050	425.0	255.0	0.020	170.0	40.0
7 a.m.–7 a.m.....	1,555	0.0510	791.6	445.3	0.022	346.3	43.8

Breakfast: Two eggs, pint of milk, bread, butter.
Lunch: Cold steak, bread, butter.
Dinner: Ham, two fried eggs, pint of milk, bread, butter, cheese.

July 11							
7 a.m.–7 p.m.....	690	0.050	345.0	145.0	0.029	200.0	58.0
7 p.m.–7 a.m.....	1,010	0.040	404.0	171.8	0.023	232.2	57.5
7 a.m.–7 a.m.....	1,700	0.0440	749.0	316.8	0.025	432.2	57.7

Breakfast: Orange, two eggs, pint of milk, bread, butter.
Lunch: Ham, bread, butter.
Dinner: Fried mackerel, one egg, pint of milk, bread, butter.

TABLE II—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 12							
7 a.m.–7 p.m.....	690	0.050	345.0	151.8	0.028	193.2	56.0
7 p.m.–7 a.m.....	1,050	0.036	378.0	157.5	0.021	220.5	58.3
7 a.m.–7 a.m.....	1,740	0.042	723.0	309.3	0.024	413.7	57.2

Breakfast: Two eggs, pint of milk, bread, butter.
Lunch: Meat wurst, bread, butter.
Dinner: Pot roast, two eggs, bread, butter, pint of milk.

sugar eliminated for this period is wholly fermentable. Indeed the absolute quantity of non-fermentable sugar drops slightly, so that the *percentage* of unfermentable sugar falls from about 60 to about 12 per cent. For the 3rd and 4th hours after breakfast the total sugar falls off markedly. This fall is wholly confined to the fermentable portion. For this period the quantity of unfermentable sugar is nearly double that of the preceding period. In each instance this increase in the unfermentable sugar is accompanied by an increase in the urinary volume. Volume, however, is not a factor in the elimination of the unfermentable sugar. A glance down the table will show that the percentage of unfermentable sugar in the urine undergoes marked changes. Furthermore, the protein diet, which yielded the largest urinary volumes gave the lowest absolute quantity of unfermentable sugar.

At present we are inclined to regard the output of the unfermentable sugar as representing a stage in digestion and absorption, or more probably a stage in the adjustment of the body to the assimilation of the sugar. When the sugar is being more adequately handled, there is an increased formation and elimination of the non-fermentable sugar.[11] This view is supported by

[11] It is of interest in this connection to note the results reported by Fisher and Wishart (*J. Biol. Chem.*, 1912–13, xiii, 49) after feeding dextrose to a fasting dog. These investigators reported that during the absorption of the sugar the blood sugar increased, accompanied by a hydremic plethora. During the 4th hour the metabolism falls to the fasting level, accompanied by a marked diuresis. This period apparently marks complete adjustment on the part of the organism to the ingested sugar.

TABLE III.

E. O.—Fermentable and non-fermentable sugar in urine on carbohydrate-rich (unweighed) diet. Collected in 2 hour periods for the day.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 16							
5 a.m.– 7 a.m.	102	0.078	79.5	26.5	0.052	53.0	66.7
7 " 9 "	85	0.34	289.0	255.0	0.04	34.0	11.8
9 " –11 "	127	0.10	127.0	67.3	0.047	59.7	47.0
11 " – 1 p.m.	100	0.088	88.0	39.0	0.049	49.0	55.7
1 p.m.– 3 "	54	0.192	103.7	64.8	0.072	38.9	37.5
3 " – 5 "	84	0.097	81.5	43.7	0.045	37.8	46.4
5 " – 7 "	40	0.117	46.8	24.4	0.056	22.4	48.0
7 " – 9 "	60	0.214	128.4	68.4	0.100	60.0	46.7
9 " –11 "	78	0.175	136.5	76.4	0.077	60.1	44.0
11 " – 5 a.m.	260	0.123	320.0	171.8	0.057	148.2	46.3
5 a.m.– 5 a.m.	990	0.141	1,400.4	837.3	0.057	563.1	40.2

Breakfast: Pancakes, syrup, bread, butter, milk, sugar, coffee.....7 a.m.–7.30 a.m.
Lunch: Crackers, milk, sugar.....1 p.m.–1.30 p.m.
Dinner: Small piece of meat, potatoes, onions, radishes, bread, butter, coffee, milk, sugar, rice pudding.....7 " –7.30 "

July 17							
5 a.m.– 7 a.m.	72	0.100	72.0	27.0	0.062	45.0	62.5
7 " – 9 "	62	0.608	377.0	331.7	0.073	45.3	12.0
9 " –11 "	110	0.163	179.3	91.3	0.08	88.0	49.1
11 " – 1 p.m.	80	0.108	86.4	50.4	0.045	36.0	41.7
1 p.m.– 3 "	60	0.182	109.2	63.3	0.076	45.9	52.0
3 " – 5 "	70	0.113	79.1	44.1	0.050	35.0	44.2
5 " – 7 "	58	0.100	58.0	23.8	0.059	34.2	59.0
7 " – 9 "	50	0.250	125.0	66.5	0.117	58.5	46.8
9 " –11 "	60	0.204	122.4	46.2	0.127	76.2	62.2
11 " – 5 a.m.	242	0.163	378.2	150.3	0.094	227.4	60.1
5 a.m.–5 a.m.	864	0.184	1,586.6	895.1	0.080	691.5	43.5

Breakfast: Pancakes, syrup, coffee, milk, sugar..7 a.m.–7.30 a.m.
Lunch: Crackers, milk, sugar.....1 p.m.–1.30 p.m.
Dinner: Small lamb chop, potatoes, lettuce, onions, bread, butter, milk, sugar, coffee, cake.....7 " –7.30 "

TABLE III—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 18							
5 a.m.– 7 a.m.....	55	0.162	89.1	30.3	0.107	58.8	66.0
7 " – 9 "	65	0.400	260.0	221.6	0.059	38.4	14.8
9 " –11 "	110	0.123	135.3	55.0	0.073	80.3	59.3
11 " – 1 p.m.....	92	0.098	90.2	29.5	0.066	60.7	67.3
1 p.m.– 3 "	65	0.25	162.5	87.8	0.115	74.7	46.0
3 " – 5 "	71	0.115	81.7	41.9	0.056	39.8	48.7
5 " – 7 "	52	0.157	81.7	38.0	0.084	43.7	53.5
7 " – 9 "	82	0.142	116.4	64.7	0.063	51.7	44.4
9 " –11 "	55	0.185	101.8	46.8	0.100	55.0	54.0
11 " – 5 a.m.....	184	0.180	331.2	158.2	0.094	173.0	52.2
5 a.m.– 5 a.m.....	831	0.175	1,449.9	773.8	0.081	676.1	46.6

Breakfast: Pancakes, syrup, coffee, milk, sugar..7 a.m.–7.30 a.m.
Lunch: Crackers, milk, sugar.....1 p.m.–1.30 p.m.
Dinner: Small piece of codfish, potatoes, lettuce, bread, butter, milk, sugar, coffee, cake.....7 " –7.30 "

experiments with another subject referred to later. In Table III it may also be noted that after the lightest meal (lunch) the maximum figure for the non-fermentable sugar is reached within the first 2 hours following, just as for the fermentable portion, while after the heaviest meal (dinner) the maximal figure for the non-fermentable sugar is reached during the 3rd and 4th hours.

The percentage of sugar in the urine on the high carbohydrate diet varies between 0.078 and 0.608 per cent. It is perhaps needless to point out that the urines containing 0.6 and 0.4 per cent of sugar gave very marked qualitative tests for sugar with the Benedict copper solution. Had these particular samples of urine happened to be examined by a clinician the conclusion would probably have been drawn that the subject was a diabetic. Almost certainly life insurance would have been refused as a result of such an examination, yet the subject has apparently no diabetic tendency. For the clinician to find such a tendency would simply require a combination of circumstances by which the urine sample should be collected within 2 hours after a breakfast

unusually rich in carbohydrate. Probably not infrequently individuals are rejected for life insurance owing to such a combination of circumstances. An accurate quantitative determination for a 24 hour sample on the ordinary diet would correct the error.

Table IV gives a detailed report of the urine excretion on an ordinary diet. The total sugar eliminated approximates closely 1 gm. per day, just as it did on the other days for a similar diet reported in Table I. The effect of breakfast on the sugar output is marked, though the figure reached is not nearly so high as for the carbohydrate diet in the preceding table. The lunch eaten at 1.30 was light and its effect is not very apparent until the hours from 3 to 5, when both glucose and unfermentable sugar reach their maximal figures for the afternoon. For each of the diets the effect of lunch is markedly less than for either of the other two meals. From other data, which we are not yet ready to report upon, we are inclined to believe that muscular activity is at least somewhat of a factor in keeping down the sugar elimination following the noonday meal. The effect of dinner, eaten at 7.30 p.m., is marked upon the sugar output until at least as late as 11 p.m. For the following hours to 7 a.m. the sugar is low, both in percentage in the urine and absolute amount per hour. Although the urinary volume is high during these 8 hours, representing about 50 per cent of the total volume, the percentage and absolute amount of sugar are decreased. Except for the night of July 4, when lobster salad composed part of the diet at dinner (which may have kept the digestive tract working for an unusually long time), the output of sugar during the resting hours is constant (256, 255, and 243 mg.) That this occurs in spite of a *wide variation in urinary volume* leads one almost to suspect that the sugar represents a definite excretory product, or in other words that there is a definite balance in the organism's power to metabolize mobilized (circulating) sugar even in the ordinary physiological quantities. From this point of view the normal organism is truly diabetic, in that it has no absolute tolerance for carbohydrate but eliminates it irrespective of urinary volume, just as does the diabetic. The difference between the normal and diabetic would then be *wholly a quantitative* one just as is that between the mild and severe diabetic. Indeed we feel that an inspection of our tables leads unmistakably to this conclusion.

TABLE IV.

E. O.—Fermentable and non-fermentable sugar in urine on ordinary diet. Urine collected in 2 hour periods from 7 a.m. to 11 p.m.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
June 29							
7 a.m.– 9 a.m.	60	0.233	139.8	88.8	0.085	51.0	36.5
9 " –11 "	80	0.090	72.0	22.4	0.062	49.6	68.9
11 " – 1 p.m.	76	0.068	51.7	16.0	0.047	35.7	69.1
1 p.m.– 3 "	54	0.109	58.9	20.0	0.072	38.9	66.0
3 " – 5 "	60	0.115	69.0	19.8	0.082	49.2	71.0
5 " – 7 "	56	0.113	63.3	15.1	0.086	48.2	76.0
7 " – 9 "	80	0.147	117.6	67.2	0.063	50.4	42.8
9 " –11 "	164	0.084	137.7	72.1	0.040	65.6	47.6
11 " – 7 a.m.	610	0.042	256.2	104.0	0.025	152.5	59.5
7 a.m.–7 a.m.	1,240	0.078	966.2	425.1	0.044	541.1	56.0

Breakfast: Force, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Bread, butter, cheese, orange.....1.30 p.m.
Dinner: Corned beef, cabbage, potatoes, pickles, bread, butter, coffee, milk, sugar.....7.30 "

June 30							
7 a.m.– 9 a.m.	100	0.130	130.0	59.0	0.071	71.0	54.6
9 " –11 "	85	0.133	96.1	45.1	0.060	51.0	53.1
11 " – 1 p.m.	72	0.086	61.9	24.5	0.052	37.4	60.4
1 p.m.– 3 "	56	0.100	56.0	35.3	0.037	20.7	37.0
3 " – 5 "	72	0.102	73.4	24.4	0.068	49.0	66.7
5 " – 7 "	64	0.125	80.0	25.6	0.085	54.4	68.0
7 " – 9 "	90	0.110	99.0	44.1	0.061	54.9	55.5
9 " –11 "	130	0.110	143.0	61.1	0.063	81.9	57.2
11 " – 7 a.m.	624	0.041	255.9	131.1	0.020	124.8	48.8
7 a.m.– 7 a.m.	1,293	0.077	995.3	450.2	0.042	545.1	54.8

Breakfast: Force, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Two eggs, bread, butter, orange.....1.30 p.m.
Dinner: Steak, potatoes, beans, bread, butter, coffee, milk, sugar, melons.....7.30 "

TABLE IV—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 4							
7 a.m.– 9 a.m.....	132	0.124	163.7	109.6	0.041	54.1	33.0
9 " –11 "	128	0.066	84.5	35.9	0.038	48.6	57.5
11 " – 1 p.m.....	95	0.061	58.0	20.5	0.040	37.5	64.6
1 p.m.– 3 "	60	0.100	60.0	19.2	0.068	40.8	68.0
3 " – 5 "	76	0.107	81.3	24.3	0.075	57.0	70.0
5 " – 7 "	64	0.107	68.5	21.1	0.074	47.4	69.2
7 " – 9 "	90	0.090	81.0	29.7	0.057	51.3	63.3
9 " –11 "	80	0.316	252.8	185.6	0.084	67.2	26.6
11 " – 7 a.m.....	500	0.071	355.0	155.0	0.04	200.0	56.3
7 a.m.– 7 a.m.....	1,225	0.098	1,204.8	600.9	0.049	603.9	50.1

Breakfast: Force, milk, sugar, bread, butter, cheese, coffee.....7.30 a.m.
Lunch: Two eggs, bread, butter, cheese, orange.....1.30 p.m.
Dinner: Lobster salad, cold meat, peas, radishes, bread, butter, milk, sugar, coffee, ice-cream.....8.00 "

July 5							
7 a.m.– 9 a.m.....	130	0.185	240.0	173.7	0.051	66.3	27.6
9 " –11 "	132	0.067	88.5	37.0	0.039	51.5	58.2
11 " – 1 p.m.....	84	0.077	65.1	19.7	0.054	45.4	69.7
1 p.m.– 3 "	58	0.099	57.5	16.9	0.070	40.6	70.6
3 " – 5 "	60	0.143	85.8	25.2	0.101	60.6	70.6
5 " – 7 "	74	0.104	77.0	21.5	0.075	55.5	72.1
7 " – 9 "	100	0.211	211.0	161.0	0.050	50.0	23.7
9 " –11 "	64	0.126	81.3	48.7	0.051	32.6	40.1
11 " – 7 a.m.....	460	0.053	243.8	105.8	0.030	138.0	56.6
7 a.m.– 7 a.m.....	1,162	0.099	1,150.0	609.5	0.046	540.5	47.0

Breakfast: Orange, peaches, krumbles, milk, sugar, bread, butter, coffee.....7.30 a.m.
Lunch: Ham, bread, butter, pretzels.....1.30 p.m.
Dinner: Fried mackerel, potatoes, lettuce, onions, bread, butter, coffee, milk, sugar, canned pears.....7.30 "

The "tolerance" of human individuals for dextrose on an empty stomach is commonly placed at about 150 gm. Taylor and Hulton[12] found practically no limit to the tolerance for orally administered dextrose in human subjects, but Myers[13] has very justly criticized the conclusions of Taylor and Hulton, and found glycosuria following the ingestion of 75 gm. of dextrose in a normal subject.

In Table V are presented results on E. O. showing the urinary sugar elimination following sugar ingestion on an empty stomach. In each experiment the hours 6 to 8 a.m. serve as control. The sugar was ingested in a measured volume of water at 8 a.m. Breakfast was eaten at 10 o'clock. For the first four experiments the quantity of dextrose ingested was 20 gm., taken in 100 cc. of water. The same quantity of water (without dextrose) was taken at 6 o'clock for the control period. The total sugar elimination for the 2 hours following the intake of the 20 gm. of dextrose shows a questionable increase over the control period, for three out of the four experiments. For one of the four experiments (August 2) there is appreciably less sugar for the 2 hours following the dextrose ingestion. In view of this fact, and in view of the fluctuations in quantity for the control periods, we should hardly conclude that E. O. shows any increased sugar elimination following an oral dose of 20 gm. of dextrose. Nevertheless, *an inspection of the table for the experimental periods shows that 20 gm. of dextrose has an unmistakable effect upon the urinary sugar*. This effect is apparent *in the changed ratio between fermentable and unfermentable sugar* following the dextrose ingestion. For the four control periods the non-fermentable sugar averages 64 per cent of the total sugar, while for the four periods following the 20 gm. of dextrose ingestion the non-fermentable sugar averages only 38 per cent of the total. And this in spite of the fact, noted above, that the quantity of *total* sugar for the different periods is only slightly altered. The same general change (lowering of percentage of non-fermentable sugar) follows food ingestion in the various tables, but in these latter cases the total sugar is materially increased. In the present experiments

[12] Taylor, A. E., and Hulton, F., *J. Biol. Chem.*, 1916, xxv, 173.

[13] Myers, V. C., *Proc. Soc. Exp. Biol. and Med.*, 1915-16, xiii, 178.

with 20 gm. of dextrose on an empty stomach we have reached a minimum of sugar which has a demonstrable effect in E. O. This effect is not upon the *quantity* of sugar eliminated, but is upon the *nature or condition* of the sugar eliminated. That a change occurs within the organism related to this change in form of excretion seems a necessary assumption. Thus there is evidence that the sudden absorption of glucose into the blood stream may alter sugar metabolism quite aside from any glycosuria in the ordinary sense. The point is probably fundamental, and will be referred to subsequently.

Although E. O. shows no glycosuria in the ordinary sense following the ingestion of 20 gm. of dextrose, an increase of this dose to 40 gm. is followed by an unmistakable increase of total sugar in the urine, as shown for August 3 and 4 in Table V. Curiously enough, the increase in sugar elimination, following 40 gm. of dextrose, for each of these experiments is exactly 100 mg. The percentage and absolute amount of unfermentable sugar fall sharply in each experiment following the ingestion of the 40 gm. of dextrose. On August 6, the dextrose ingested was increased to 60 gm. The urinary sugar increases by about 400 mg. over the control period, and the percentage of unfermentable sugar in the urine falls from 57 to 5 per cent for the 2 hours following the sugar ingestion. The effect of the 60 gm. of dextrose is plainly apparent in the 24 hour urine, yet the *percentage* of sugar in this total urine is scarcely higher than that on August 2, where the absolute sugar elimination was about 0.5 gm. less. This result serves to emphasize the inherent error in determining glycosuria through percentage of sugar in the urine, or through a qualitative test of an individual sample.

Before concluding this discussion of the effect of glucose ingestion on an empty stomach it may be of interest to call attention to one further point of possible therapeutic application in connection with diabetes. This point will require further work to establish it, but the present results are at least suggestive. In the experiments recorded in Table V the breakfast eaten was not weighed, but it practically duplicated that eaten for earlier experiments as recorded in Table IV. Yet we note that in Table V, following the ingestion of the glucose on an empty stomach, the increase in the sugar of the urine which follows breakfast is not

TABLE V.

E. O.—Experiments to test the effect on the urinary sugar of varying amounts of sugars ingested on an empty stomach. 6 to 8 a.m. is taken as the control period in each experiment. Details of dextrose ingested and subsequent diet are given following the figures for each day.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 20							
6 a.m.– 8 a.m.....	100	0.079	79.0	22.0	0.057	57.0	72.2
8 " –10 "	110	0.090	99.0	67.1	0.029	31.9	32.2
10 " –12 "	150	0.093	139.5	76.5	0.042	63.0	45.1
12 noon– 2 p.m.....	132	0.056	74.0	23.8	0.038	50.2	67.8
2 p.m.– 6 "	140	0.125	175.0	56.0	0.085	119.0	68.0
6 " – 6 a.m.....	274	0.192	522.1	278.1	0.089	244.0	46.7
6 a.m.– 6 a.m.....	906	0.12	1,088.6	523.5	0.062	565.1	51.9

6 a.m., drank 100 cc. water.
8 " 100 cc. water + 20 gm. dextrose.
Breakfast: Corn flakes, milk, sugar, bread, butter, coffee..... 10.00 a.m.
Lunch: Bread, butter, cheese, dried beef..... 2.00 p.m.
Dinner: Corned beef, cabbage, potatoes, sweet corn, bread, butter, milk, sugar, coffee, peaches, cream.... 7.20 "

Aug. 21							
6 a.m.– 8 a.m.....	74	0.091	67.3	22.9	0.06	44.4	66.0
8 " –10 "	90	0.086	77.4	38.7	0.043	38.7	50.0
10 " –12 "	64	0.142	90.9	37.8	0.083	53.1	58.4
12 noon– 2 p.m....	74	0.116	85.8	36.2	0.067	49.6	57.8
2 p.m.– 6 "	140	0.115	161.0	67.2	0.067	93.8	58.3
6 " – 6 a.m.....	462	0.116	536.0	305.0	0.05	231.0	43.1
6 a.m.– 6 a.m.....	904	0.113	1,018.4	507.8	0.056	510.6	50.2

6 a.m., 100 cc. water.
8 " 100 " " + 20 gm. dextrose.
Breakfast: Corn flakes, milk, sugar, bread, butter, cheese..... 10.00 a.m.
Lunch: Bread, butter, corned beef, cheese..... 2.00 p.m.
Dinner: Beef stew, lima beans, potatoes, corn bread, butter, milk, sugar, tea, cake..... 7.30 "

TABLE V—*Continued.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 22							
6 a.m.– 8 a.m.	86	0.068	58.5	20.7	0.044	37.8	64.7
8 " –10 "	88	0.072	63.4	38.8	0.028	24.6	38.8
10 " –12 "	82	0.133	109.1	68.1	0.05	41.0	37.6
12 noon– 2 p.m.	100	0.077	77.0	39.0	0.038	38.0	49.4
2 p.m.– 6 "	138	0.117	162.0	65.4	0.070	96.6	59.6
6 " – 6 a.m.	446	0.117	522.0	263.3	0.058	258.7	49.5
6 a.m.– 6 a.m.	940	0.1056	992.0	495.3	0.053	496.7	50.1

6 a.m., 100 cc. water.
8 " 100 " " + 20 gm. dextrose.
Breakfast: Corn flakes, milk, sugar, bread, butter, egg, cheese.....10.00 a.m.
Lunch: Bread, butter, ham, cheese..... 2.00 p.m.
Dinner: Steak, peas, potatoes, tomatoes, bread, butter, milk, coffee, sugar, apple pie..... 7.30 "

Aug. 2.							
6 a.m.– 8 a.m.	70	0.109	76.3	32.9	0.062	43.4	57.0
8 " –10 "	48	0.128	61.5	40.9	0.043	20.6	33.5
10 " –12 "	96	0.102	97.9	54.7	0.045	43.2	44.1
12 noon– 2 p.m.	110	0.076	83.6	38.5	0.041	45.1	54.0
2 p.m.– 6 "	162	0.115	186.3	71.3	0.071	115.0	61.7
6 " – 6 a.m.	305	0.171	521.6	204.5	0.104	317.1	60.8
6 a.m.– 6 a.m.	791	0.130	1,027.2	442.8	0.074	584.4	56.9

6 a.m., 100 cc. water.
8 " 100 " " + 20 gm. dextrose.
Breakfast: Corn flakes, milk, sugar, two eggs, bread, butter, coffee, orange.....10.00 a.m.
Lunch: Bread, butter, cheese, meat wurst..... 2.00 p.m.
Dinner: Hamburger steak, potatoes, beets, lettuce, lima beans, bread, butter, coffee, milk, sugar, ice-cream, peaches, cookies..... 6.00 "

TABLE V—*Continued.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 3							
6 a.m.– 8 a.m.	60	0.112	67.2	24.0	0.077	43.2	64.3
8 " –10 "	46	0.365	167.9	126.5	0.090	41.4	24.7
10 " –12 "	90	0.125	112.5	56.7	0.062	55.8	49.6
12 noon– 2 p.m.	85	0.075	63.8	22.1	0.049	41.7	65.4
2 p.m.– 6 "	122	0.136	165.9	98.2	0.055	67.7	40.8
6 " – 6 a.m.	550	0.092	506.1	330.1	0.032	176.0	34.8
6 a.m.– 6 a.m.	953	0.114	1,083.4	657.6	0.045	425.8	39.3

6 a.m., 100 cc. water
8 " 100 " " + 40 gm. dextrose.
Breakfast: Orange, corn flakes, milk, sugar, coffee, one egg, bread, butter..... 10.00 a.m.
Lunch: Bread, butter, ham..... 2.00 p.m.
Dinner: Lamb stew, potatoes, onions, lettuce, bread, butter, milk, sugar, tea, cake..... 6.00 "

Aug. 4							
6 a.m.– 8 a.m.	110	0.083	91.3	51.7	0.036	39.6	43.4
8 " –10 "	68	0.282	191.8	163.2	0.042	28.6	14.9
10 " –12 "	88	0.111	97.7	59.9	0.043	37.8	38.7
12 noon– 2 p.m.	102	0.067	68.4	39.8	0.028	28.6	41.8
2 p.m.– 6 "	148	0.104	154.0	75.5	0.053	78.5	51.0
6 " 6 a.m.	610	0.087	530.0	280.0	0.041	250.0	47.2
6 a.m.– 6 a.m.	1,126	0.100	1,133.2	670.1	0.041	463.1	40.9

6 a.m., 150 cc. water.
8 " 150 " " + 40 gm. dextrose.
Breakfast: Orange, corn flakes, milk, sugar, one egg, bread, butter, coffee..... 10.00 a.m.
Lunch: Bread, butter, cheese, meat wurst..... 2.00 p.m.
Dinner: Boiled ham, creamed potatoes, beets, macaroni, bread, butter, milk, sugar, tea, cake..... 6.30 "

TABLE V—*Continued.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 6							
6 a.m.– 8 a.m.....	180	0.04	72.0	30.6	0.023	41.4	57.5
8 " –10 "	116	0.50	580.0	548.7	0.027	31.3	5.4
10 " –12 "	126	0.083	104.6	76.9	0.022	27.7	26.5
12 noon– 2 p.m....	110	0.064	70.4	56.1	0.013	14.3	20.3
2 p.m.– 6 "	80	0.244	195.2	112.8	0.103	82.4	42.2
6 " – 4 a.m.....	405	0.12	486.0	149.8	0.083	336.2	69.2
4 a.m.– 6 "	60	0.134	80.4	24.0	0.094	56.4	70.2
6 a.m.– 6 a.m.....	1,077	0.147	1,588.6	998.9	0.055	589.7	37.1

6 a.m., 150 cc. water.
8 " 150 " " + 60 gm. dextrose.
Breakfast: Orange, corn flakes, milk, sugar, one egg, bread, butter, coffee.....10.00 a.m.
Lunch: Bread, butter, ham, cheese..... 2.30 p.m.
Dinner: Veal loaf, potatoes, tomatoes, cucumber, macaroni, bread, butter, milk, sugar, coffee..... 6.00 "

E. O.—Experiments to test the effect on the urinary sugar output of 20 gm. of cane sugar ingested on an empty stomach. The hours 6 to 8 a.m. are used as control.

Aug. 23							
6 a.m.– 8 a.m.....	76	0.10	76.0	27.4	0.064	48.6	64.0
8 " –10 "	118	0.07	82.6	38.9	0.037	43.7	52.9
10 " –12 "	106	0.107	113.4	66.8	0.044	46.6	41.1
12 noon–2 p.m.....	124	0.063	78.1	38.4	0.032	39.7	50.8
2 p.m.– 6 "	122	0.138	168.4	70.8	0.080	97.6	58.0
6 " – 6 a.m.....	820	0.063	516.6	270.6	0.03	246.0	47.3
6 a.m.– 6 a.m.....	1,366	0.076	1,035.1	512.9	0.038	522.2	50.5

6 a.m., 100 cc. water.
8 " 100 " " + 20 gm. cane sugar.
Breakfast: Corn flakes, milk, sugar.....10.00 a.m.
Lunch: Bread, butter, ham, cheese..... 2.00 p.m.
Dinner: Hamburger steak, potatoes, cucumber, pickle, bread, butter, milk, sugar, tea, pears, cake..... 6.00 "

TABLE V—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 24							
6 a.m.– 8 a.m.	120	0.062	74.4	22.8	0.043	51.6	69.3
8 " –10 "	106	0.077	81.6	41.3	0.038	40.3	49.4
10 " –12 "	115	0.088	101.2	62.1	0.034	39.1	38.7
12 noon– 2 p.m.	144	0.075	108.0	60.5	0.033	47.5	44.0
2 p.m.– 6 "	224	0.082	183.7	103.1	0.036	80.6	43.9
6 " – 5 a.m.	610	0.077	469.6	256.1	0.035	213.5	45.5
5 a.m.– 6 "	56	0.081	45.3	20.1	0.045	25.2	55.6
6 a.m.– 6 a.m.	1,375	0.077	1,063.8	566.0	0.036	497.8	46.8

6 a.m., 100 cc. water.
8 " 100 " " + 20 gm. cane sugar.
Breakfast: Corn flakes, milk, sugar ..... 10.00 a.m.
Lunch: Two slices of bread, butter, milk, salmon, meat wurst ..... 2.00 p.m.
Dinner: Beef steak, potatoes, onions, lettuce, bread, butter, milk, sugar, coffee, ice-cream, cookies, watermelon ..... 6.30 "

nearly so great as where (as in Table IV) the preliminary glucose was not taken. To just what this difference is due will have to be determined by subsequent work, where the breakfasts are weighed, and where the muscular activity is noted. Nevertheless, a view which suggests itself is that preliminary ingestion of pure dextrose may act as a stimulant for internal pancreatic function, so that assimilation of sugar is better following the subsequent meal.

Table V concludes with two experiments on the ingestion of 20 gm. of cane sugar on an empty stomach. There is a slight increase apparent in the total sugar output, but further experiments would be necessary to eliminate these increases from the possibility of natural fluctuations. As in the case of the 20 gm. of glucose, there is a definite fall in the percentage of unfermentable sugar, but this fall is not nearly so marked as in the experiments with a similar quantity of dextrose. Subsequent experiments show that cane sugar is not so effective in disturbing carbohydrate

metabolism, especially during digestion, as is dextrose (see Table VI).

Table VI records experiments with E. O. in which 20 gm. of dextrose are added to a weighed breakfast. One day is used as control in each experiment, and in interpreting the effect of the added sugar (taken with breakfast at 7 a.m.) comparison should be made with the same hours of the day preceding. In the discussion of Table V it was noted that 20 gm. of glucose taken by E. O. on an empty stomach caused no appreciable increase in the *total* sugar eliminated, but did cause a marked fall in absolute amount and *percentage* of non-fermentable sugar. Table VI shows that addition of 20 gm. of dextrose to a weighed breakfast causes a very marked increase in the total sugar eliminated and a sharp drop in the percentage of non-fermentable sugar. Thus the control days show that the weighed breakfast alone was followed in the next 2 hours by an elimination of 115 to 127 mg. of total sugar, about 50 per cent of which was non-fermentable. When 20 gm. of dextrose was added to this same breakfast the total sugar elimination for the following 2 hours reached 375 to 400 mg., of which only 7 to 13 per cent was unfermentable. The weighed breakfast contained some sugar (cane sugar and lactose) and it might be objected that the strikingly greater effect of the glucose added to breakfast than when taken on an empty stomach was due to the presence of this additional sugar in the diet. That this is not so is shown by the experiments recorded in Table VII, where the same weighed breakfast used in the dextrose experiments was employed, but where 20 gm. of cane sugar were added. The cane sugar causes *no* increase in one of the two experiments and a questionable increase in the other. The percentage of unfermentable sugar in the urine falls following the cane sugar ingestion, irrespective of any change in the total sugar.

The very high sugar elimination reached as a result of addition of 20 gm. of dextrose to a meal (breakfast) as recorded in Table VI appears to lend considerable support to the view held by several students of diabetes that the internal function of the pancreas may suffer while the gland is active during digestion. Unless some such view is taken it is difficult to understand why, as in our experiments, dextrose added to a meal is more poorly tolerated than when taken on an empty stomach.

TABLE VI.

E. O.—Experiments to test the effect on the urinary sugar output of 20 gm. of dextrose added to a weighed breakfast.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 25							
5 a.m.– 7 a.m....	100	0.081	81.0	32.0	0.049	49.0	60.5
7 " – 9 "	92	0.138	127.0	57.0	0.076	70.0	55.1
9 " –11 "	114	0.080	91.2	37.6	0.047	53.6	58.8
11 " – 1 p.m....	92	0.069	63.5	22.1	0.045	41.4	65.2
1 p.m.– 3 "	66	0.143	94.4	55.4	0.059	39.0	42.3
3 " – 5 "	80	0.110	88.0	47.2	0.051	40.8	46.4
5 " – 7 "	68	0.129	87.7	39.4	0.071	48.3	55.0
7 " – 9 "	66	0.178	117.5	60.1	0.087	57.4	49.0
9 " –11 "	64	0.123	78.7	32.6	0.072	46.1	58.5
11 " – 5 a.m.....	452	0.058	262.1	108.4	0.034	153.7	58.6
5 a.m.– 5 a.m.....	1,194	0.091	1,091.1	491.8	0.050	599.3	55.0

Breakfast: 100 gm. oranges, 25 gm. corn flakes, 30 gm. sugar, 40 gm. bread, 15 gm. butter, 150 gm. milk.....7.10 a.m.
Lunch: Bread, butter, milk, meat wurst, smoked ham....1.15 p.m.
Dinner: Corned beef, baked beans, potatoes, lettuce, bread, butter, milk, sugar, coffee, cake.....7.20 "

Aug. 26							
5 a.m.– 7 a.m.....	70	0.096	67.2	22.4	0.064	44.8	66.7
7 " – 9 "	62	0.65	403.0	373.8	0.047	29.2	7.3
9 " –11 "	112	0.089	99.7	53.8	0.041	45.9	46.1
11 " – 1 p.m.....	80	0.075	60.0	24.8	0.044	35.2	58.7
1 p.m.– 3 "	80	0.133	106.4	51.2	0.069	55.2	51.9
3 " – 5 "	78	0.092	71.8	25.0	0.06	46.8	65.2
5 " – 7 "	86	0.10	86.0	31.8	0.063	54.2	63.0
7 " – 9 "	70	0.155	108.5	58.1	0.072	50.4	46.4
9 " –11 "	72	0.145	104.4	44.6	0.083	59.8	57.3
11 " – 5 a.m.....	420	0.073	306.5	125.9	0.043	180.6	59.0
5 a.m.– 5 a.m.....	1,130	0.125	1,413.5	811.4	0.053	602.1	42.6

Breakfast: Same as Aug. 25 + 20 gm. dextrose..... 7.00 a.m.
Lunch: Bread, butter, egg, cheese, milk..... 1.00 p.m.
Dinner: Veal roast, potatoes, carrots, olives, turnips, peas, bread, butter, milk, sugar, coffee, apple cake... 7.15 "

TABLE VI—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 27							
5 a.m.– 7 a.m.	140	0.046	64.4	25.2	0.028	39.2	60.9
7 " – 9 "	120	0.097	116.4	55.2	0.051	61.2	52.6
9 " –11 "	120	0.054	64.8	25.2	0.033	39.6	61.1
11 " – 1 p.m.	74	0.077	57.0	21.5	0.048	35.5	62.3
1 p.m.– 3 "	60	0.135	81.0	48.0	0.055	33.0	40.8
3 " – 5 "	58	0.125	72.5	37.7	0.06	34.8	48.0
5 " – 7 "	70	0.108	75.6	32.2	0.062	43.4	57.4
7 " – 9 "	100	0.106	106.0	49.0	0.057	57.0	53.8
9 " –11 "	96	0.098	94.1	43.2	0.053	50.9	54.1
11 " – 5 a.m.	340	0.101	343.4	139.4	0.06	204.0	59.4
5 a.m.– 5 a.m.	1,178	0.091	1,075.2	476.6	0.051	598.6	55.7

Breakfast: Same as Aug. 25 ..... 7.00 a.m.
Lunch: Butter, milk, meat, cheese ..... 1.00 p.m.
Dinner: Roast pork, potatoes, peas, lima beans, pickles, bread, butter, milk, sugar, coffee, banana pudding ..... 7.15 "

Aug. 28							
5 a.m.– 7 a.m.	64	0.102	65.3	21.2	0.069	44.1	67.5
7 " – 9 "	70	0.535	374.5	324.1	0.072	50.4	13.5
9 " –11 "	120	0.071	85.2	40.8	0.037	44.4	52.1
11 " – 1 p.m.	76	0.079	60.0	23.5	0.048	36.5	60.8
1 p.m.– 3 "	54	0.146	78.9	42.7	0.067	36.2	45.9
3 " – 5 "	70	0.142	99.4	39.2	0.086	60.2	60.5
5 " – 7 "	80	0.103	82.4	31.2	0.064	51.2	62.1
7 " – 9 "	76	0.165	125.4	72.2	0.07	53.2	42.4
9 " –11 "	84	0.110	92.4	44.5	0.057	47.9	51.8
11 " – 5 a.m.	378	0.084	317.5	136.1	0.048	181.4	57.1
5 a.m.– 5 a.m.	1,072	0.128	1,381.0	775.5	0.056	605.5	43.8

Breakfast: Same as Aug. 25 + 20 gm. dextrose ..... 7.00 a.m.
Lunch: Bread, butter, milk, ham, cheese ..... 1.30 p.m.
Dinner: Cooked salmon, potatoes, salad, cucumbers, bread, butter, coffee, milk, sugar, peach jelly, cream ..... 7.00 "

TABLE VII.

E. O.—Experiment to test the effect of 20 gm. of cane sugar ingested with a weighed breakfast. The first day is used as a control in each experiment.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
Aug. 29							
5 a.m.– 7 a.m....	78	0.105	81.9	28.1	0.069	53.8	65.7
7 " – 9 "	126	0.10	126.0	60.5	0.052	65.5	52.0
9 " –11 "	150	0.058	87.0	43.5	0.029	43.5	50.0
11 " – 1 p.m....	94	0.067	63.0	20.7	0.045	42.3	67.1
1 p.m – 3 "	70	0.112	78.4	35.0	0.062	43.4	55.4
3 " – 5 "	80	0.092	73.6	30.4	0.054	43.2	58.7
5 " – 7 "	95	0.079	75.0	30.3	0.047	44.7	59.6
7 " – 9 "	80	0.136	108.8	57.6	0.064	51.2	47.1
9 " –11 "	90	0.098	88.2	37.8	0.056	50.4	57.1
11 " – 5 a.m....	248	0.10	248.0	96.7	0.061	151.3	61.0
5 a.m.– 5 a.m....	1,111	0.092	1,029.9	440.6	0.053	589.3	57.2

Breakfast: Same as Aug. 25..... 7.15 a.m.
Lunch: Bread, butter, cheese, milk..... 1.15 p.m.
Dinner: Hamburger steak, onions, potatoes, corn, tomatoes, bread, butter, milk, sugar, coffee, crackers, cheese..... 7.00 "

Aug. 30							
5 a.m.– 7 a.m....	94	0.073	68.6	26.3	0.045	42.3	58.0
7 " – 9 "	112	0.103	115.4	71.7	0.029	43.7	37.9
9 " –11 "	136	0.048	65.3	28.6	0.027	36.7	56.2
11 " – 1 p.m....	104	0.057	59.3	21.9	0.036	37.4	63.1
1 p.m.– 3 "	84	0.108	90.7	55.4	0.042	35.3	38.9
3 " – 5 "	80	0.079	63.2	24.8	0.048	38.4	60.8
5 " – 7 "	92	0.076	69.9	28.5	0.045	41.4	59.2
7 " – 9 "	86	0.173	148.7	94.5	0.063	54.2	36.7
9 " –11 "	100	0.098	98.0	48.0	0.05	50.0	51.0
11 " – 5 a.m....	442	0.062	274.0	97.2	0.04	176.8	64.5
5 a.m.– 5 a.m....	1,330	0.792	1,053.1	496.9	0.041	556.2	52.8

Breakfast: Same as Aug. 25 + 20 gm. cane sugar..... 7.10 a.m.
Lunch: Bread, butter, milk, cold meat, cheese ..... 1.00 p.m.
Dinner: Veal cutlets, potatoes, lettuce, tomatoes, bread, butter, milk, sugar, tea, pears, ice-cream, cake..... 7.00 "

TABLE VII—*Concluded.*

Aug. 31							
5 a.m.– 7 a.m.	86	0.078	67.1	27.5	0.046	39.6	59.0
7 " – 9 "	92	0.120	110.0	59.4	0.055	50.6	46.0
9 " –11 "	138	0.066	91.1	51.1	0.029	40.0	44.0
11 " – 1 p.m.	96	0.06	57.6	21.1	0.038	36.5	63.4
1 p.m.– 3 "	60	0.143	85.8	57.6	0.047	28.2	32.9
3 " – 5 "	72	0.115	82.8	36.0	0.065	46.8	56.5
5 " – 7 "	80	0.072	57.6	21.6	0.045	36.0	62.5
7 " – 9 "	78	0.123	96.0	48.4	0.061	47.6	49.6
9 " –11 "	86	0.089	76.5	30.1	0.054	46.4	60.6
11 " – 5 a.m.	294	0.124	31.4	149.9	0.073	214.6	58.9
5 a.m.– 5 a.m.	1,082	0.100	1,089.0	502.7	0.054	586.3	53.8

Breakfast: Same as Aug. 25 ..... 7.10 a.m.
Lunch: Bread, butter, milk, salmon, cheese ..... 1.15 p.m.
Dinner: Boiled haddock, potatoes, olives, cucumbers, bread, butter, milk, sugar, coffee, cake ..... 7.30 "

Sept. 1							
5 a.m.– 7 a.m.	90	0.092	82.8	33.3	0.055	49.5	59.8
7 " – 9 "	64	0.214	137.0	96.0	0.064	41.0	30.0
9 " –11 "	125	0.084	105.0	53.7	0.041	51.3	48.9
11 " – 1 p.m.	80	0.078	62.4	21.6	0.051	40.8	65.4
1 p.m.– 3 "	93	0.078	72.6	33.5	0.042	39.1	53.9
3 " – 5 "	76	0.075	57.0	22.0	0.046	35.0	61.4
5 " – 7 "	86	0.066	56.8	18.1	0.045	38.7	68.1
7 " – 9 "	80	0.148	118.4	61.6	0.071	56.8	48.0
9 " –11 "	90	0.092	82.8	35.1	0.053	47.7	57.6
11 " – 5 a.m.	500	0.045	225.0	90.0	0.027	135.0	60.0
5 a.m.– 5 a.m.	1,284	0.077	999.8	464.9	0.041	534.9	53.5

Breakfast: Same as Aug. 25 + 20 gm. cane sugar ..... 7.00 a.m.
Lunch: Bread, butter, milk, ham, cheese ..... 1.00 p.m.
Dinner: Corned beef, cabbage, baked beans, potatoes, bread, butter, milk, sugar, coffee ..... 7.00 "

The point is further emphasized in Table VIII. In the experiment there recorded the subject was on an accurately weighed diet (nearly free from sugar) for 3 days, and the sugar elimination observed. During the 2 following days the same diet was continued, but 25 gm. of dextrose was added to each meal. The effect of even this small quantity of dextrose *taken with each meal*

TABLE VIII.

E. O.—Experiments to test the effect of adding 25 gm. of dextrose to each meal (weighed diet). The first 3 days serve as control.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 23							
7 a.m.– 7 p.m....	490	0.095	465.4	274.3	0.039	191.1	41.1
7 p.m.– 5 a.m....	795	0.056	445.2	262.4	0.023	182.8	41.1
7 a.m.– 5 a.m....	1,285	0.071	910.6	536.7	0.029	373.9	41.1
July 24							
5 a.m.– 7 a.m....	110	0.069	75.9	26.4	0.045	49.5	65.2
7 " – 7 p.m....	410	0.111	455.1	254.1	0.049	201.0	44.2
7 p.m.– 5 a.m....	565	0.071	401.2	164.0	0.042	237.2	59.1
5 a.m.– 5 a.m....	1,085	0.086	932.2	444.5	0.045	487.7	52.3
July 25							
5 a.m.– 7 a.m....	80	0.084	67.2	17.6	0.062	49.6	73.8
7 " – 7 p.m....	400	0.125	500.0	272.0	0.057	228.0	45.6
7 p.m.– 5 a.m....	500	0.079	395.0	195.0	0.040	200.0	50.7
5 a.m.– 5 a.m....	980	0.098	962.2	484.6	0.049	477.6	49.7

July 23, 24, 25.

Breakfast: Corn flakes, 40 gm., milk, 480 gm., one egg ..... 7 a.m.–7.30 a.m.

Lunch: Crackers, 60 gm., milk, 487 gm., orange, 100 gm ..... 1 p.m.–1.30 p.m.

Dinner: Bread, 50 gm., butter, 20 gm., meat, 40 gm., sugar, 10 gm., two eggs, cup of coffee 7 " 7.30 "

July 26							
5 a.m.– 7 a.m....	58	0.109	63.2	22.6	0.07	40.6	64.2
7 " – 7 p.m....	565	0.318	1,796.0	1,643.5	0.027	152.5	8.5
7 p.m.– 5 a.m....	445	0.111	494.0	396.1	0.022	97.9	19.8
5 a.m.– 5 a.m....	1,068	0.220	2,353.2	2,062.2	0.027	291.0	12.4

Same diet + 25 gm. dextrose with each meal.

TABLE VIII—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 27							
5 a.m.– 7 a.m....	78	0.077	60.1	38.3	0.028	21.8	36.3
7 " – 7 p.m....	435	0.300	1,305.0	1,161.4	0.033	143.6	11.0
7 p.m.– 5 a.m....	1,350	0.061	823.4	647.9	0.013	175.5	21.3
5 a.m.– 5 a.m....	1,863	0.117	2,188.5	1,847.6	0.018	340.9	15.6

Same diet + 25 gm. dextrose with each meal.

is truly remarkable. The carbohydrate metabolism is profoundly disturbed, as illustrated by an elimination of total sugar for the experimental days which is more than twice as great as on the control days. Furthermore the non-fermentable sugar decreases in absolute amount, and falls from a percentage of 40 to 50 on the control days to an average of 12 and 15 per cent for the experimental days.

Several of the results recorded for E. O. might lead the reader to conclude that he is a diabetic of at least a mild type. In reply to such an interpretation it should be pointed out that the method of study we have employed is essentially new, and the results render necessary a reconsideration of past conclusions concerning carbohydrate "tolerance." E. O. has been examined for years before, and for many months after these experiments with no indication, by ordinary qualitative methods, of a low carbohydrate tolerance.

Tables IX to XIV present the results of investigations on the urinary sugar output of a second subject, I. N. The experiments were planned along the same lines as those reported for E. O. For this reason the tables will not require such detailed comment as was necessary in calling attention to the results obtained for E. O.

Tables IX, X, and XI present the urinary sugar findings for I. N. upon ordinary, carbohydrate-poor, and carbohydrate-rich diets. The effects of these diets parallel quite closely the similar experiments with E. O. On the ordinary diet I. N. eliminates between 608 and 697 mg. of total sugar per day for the 3

TABLE IX.

I. N.—Fermentable and non-fermentable sugar in urine on ordinary (unweighed) mixed diet.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 5							
7 a.m.– 9 a.m.....	116	0.036	41.7	22.8	0.016	18.9	45.3
9 " –11 "	116	0.046	53.6	26.6	0.023	27.0	50.4
11 " – 1 p.m.....	150	0.025	38.4	15.3	0.015	23.1	60.1
1 p.m.– 3 "	84	0.070	59.1	30.8	0.033	28.3	47.9
3 " – 5 "	76	0.103	78.4	34.0	0.058	44.4	56.6
5 " – 7 "	64	0.078	50.3	17.7	0.051	32.6	64.8
7 " – 9 "	64	0.113	72.3	40.0	0.050	32.3	44.5
9 " –11 "	69	0.110	75.9	35.1	0.059	40.8	53.8
11 " – 7 a.m.....	345	0.041	142.9	49.7	0.027	93.2	65.3
7 a.m.– 7 a.m.....	1,084	0.056	612.6	272.0	0.031	340.6	55.6

Breakfast: Roll, egg, milk, butter..... 8.00 a.m.
Lunch: Two rolls, Swiss cheese, butter, orange, apricot.. 1.00 p.m.
Supper: Bread, salmon, butter, onions, milk, malted milk..... 7.10 "

July 6							
7 a.m.– 9 a.m.....	140	0.028	40.5	18.1	0.016	22.4	55.4
9 " –11 "	72	0.065	47.4	20.1	0.037	27.3	57.5
11 " – 1 p.m.....	85	0.046	39.3	13.6	0.030	25.7	65.4
1 p.m.– 3 "	70	0.086	60.3	27.9	0.046	32.4	53.8
3 " – 5 "	60	0.126	75.6	36.5	0.065	39.1	51.7
5 " – 7 "	86	0.064	55.2	19.9	0.041	35.3	64.0
7 " – 9 "	68	0.141	95.9	58.4	0.055	37.5	39.1
9 " –11 "	75	0.127	95.4	47.8	0.063	47.6	50.0
11 " – 7 a.m.....	345	0.054	188.0	76.2	0.032	111.8	59.4
7 a.m.– 7 a.m.....	1,001	0.069	697.6	318.5	0.037	379.1	54.3

Breakfast: Roll, egg, milk, butter..... 8.00 a.m.
Lunch: Two rolls, chicken liver, orange, apple, peach.. 1.00 p.m.
Supper: Ice-cream, wheat bread, fish, chicken, balled meat, cooked cherries..... 7.25 "

TABLE IX—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 9							
7 a.m.– 9 a.m.	86	0.048	41.8	18.9	0.026	22.9	54.7
9 " –11 "	70	0.070	49.2	22.7	0.037	26.5	53.9
11 " – 1 p.m.	90	0.047	42.5	17.6	0.027	24.9	58.7
1 p.m.– 3 "	74	0.074	55.4	25.4	0.040	30.0	54.1
3 " – 5 "	64	0.109	69.8	30.4	0.061	39.4	56.5
5 " – 7 "	106	0.046	49.5	18.1	0.029	31.4	63.4
7 " – 9 "	96	0.066	64.0	35.9	0.029	28.1	43.9
9 " –11 "	70	0.096	67.4	29.8	0.053	37.6	55.7
11 " – 7 a.m.	300	0.056	168.6	52.7	0.038	115.9	68.2
7 a.m.– 7a.m.	956	0.063	608.2	251.5	0.037	356.7	58.6

Breakfast: Egg, roll, milk, butter ..... 8.05 a.m.
Lunch: Two rolls, minster cheese, butter, orange, peach, tomato ..... 1.15 p.m.
Supper: Bread, cream cheese, onions, milk, malted milk ..... 7.20 "

days reported, as against about 1 gm. for E. O. The non-fermentable sugar makes up from 54 to 58 per cent of the total sugar on this diet. The sugar increase following food ingestion is to be noted as in the case of E. O., but the rise is not so prompt nor so marked. Throughout all the tables for I. N. it will be seen that his sugar tolerance is markedly greater than that of E. O. It should be noted in this connection that I. N. is young, muscular, and a light eater.

On a high protein diet (Table X) I. N. shows the marked drop in total sugar elimination noted for the preceding subject. This drop is not apparent until after the first day. The non-fermentable sugar also drops, but not so much as does the fermentable, so that the percentage of the non-fermentable sugar is appreciably higher on the carbohydrate-poor diet.

On the high carbohydrate diet (Table XI) I. N. shows a rise in both fermentable and unfermentable sugar. The total sugar excretion reaches 1.5 gm. on this diet. The immediate effect of

TABLE X.

I. N.—Fermentable and non-fermentable sugar in urine on carbohydrate-poor (high protein) diet. Urine collected in 12 hour periods (7 a.m. to 7 p.m. to 7 a.m.). Details of diet for the various days are given in the table.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
June 28							
Day	580	0.049	287.1	121.2	0.028	165.9	57.8
Night	520	0.082	429.5	210.5	0.042	219.0	51.0
	1,100	0.065	716.6	331.7	0.035	384.9	53.7
June 29							
Day	540	0.044	239.2	56.7	0.033	182.5	76.3
Night	590	0.048	283.2	98.5	0.031	184.7	65.2
	1,130	0.042	522.4	155.2	0.032	367.2	70.3
June 30							
Day	510	0.047	240.7	85.7	0.030	155.0	64.4
Night	480	0.055	266.4	78.2	0.039	188.2	70.6
	990	0.051	507.1	163.9	0.034	343.2	67.3
July 1							
Day	810	0.032	260.0	76.9	0.022	183.1	70.4
Night	430	0.054	235.6	55.4	0.041	180.2	76.5
	1,240	0.040	495.0	132.3	0.029	363.3	73.3
July 2							
Day	690	0.036	250.4	113.1	0.020	137.3	54.8
Night	430	0.059	257.6	92.0	0.038	165.6	64.3
	1,120	0.045	508.0	205.1	0.027	302.9	59.6
July 3							
Day	430	0.060	260.6	92.4	0.039	168.2	64.5
Night	490	0.051	253.4	105.4	0.030	148.0	58.4
	920	0.056	514.0	197.8	0.034	316.2	61.5
July 4							
Day	385	0.059	228.4	77.5	0.039	150.9	66.1
Night	380	0.059	226.9	47.6	0.047	179.3	79.0
	765	0.059	455.3	125.1	0.043	330.2	72.5

TABLE X—*Concluded.*

Date.		Diet.	
June 28			
	Breakfast...	Two eggs, milk, one-half roll, butter.	8.10 a.m.
	Lunch.....	One-half roll, corned beef, mustard, orange.	12.30 p.m.
	Supper.....	Bread (one-half slice), corned beef, cucumbers.	7.05 "
June 29			
	Breakfast...	One-half roll, butter, two eggs, milk.	8.05 a.m.
	Lunch.....	Chicken liver, one-half roll, one-half orange.	12.00 p.m.
	Supper.....	Milk (7 p.m.), fish, chicken, boiled meat, white bread (one-half slice).	8.00 "
June 30			
	Breakfast...	Bread (one-half slice), butter, milk, two eggs.	8.00 a.m.
	Lunch.....	Bread (one-half slice), chicken, stuffed neck.	12.05 p.m.
	Supper.....	White bread (one-half slice), chicken, fish.	7.00 "
July 1			
	Breakfast...	One-half roll, butter, milk, two eggs.	8.15 a.m.
	Lunch.....	White bread (one-half slice), steak.	12.50 p.m.
	Supper.....	Bread (one-half slice), three frankfurters, mustard.	7.05 "
July 2			
	Breakfast...	One-half roll, two eggs, butter, milk.	8.10 a.m.
	Lunch.....	" " corned beef, mustard.	12.25 p.m.
	Supper.....	Bread (one-half slice), breast beef.	7.05 "
July 3			
	Breakfast...	One-half roll, butter, two eggs, milk.	8.10 a.m.
	Lunch.....	" " rolled beef, mustard.	12.10 p.m.
	Supper.....	Bread (one-half slice), cutlets.....	7.05 "
July 4			
	Breakfast...	One-half roll, butter, two eggs, milk.	8.05 a.m.
	Lunch.....	" " corned beef, mustard.	12.10 p.m.
	Supper.....	Hamburger steak (four), peas.....	7.10 "

Bread unless otherwise stated signifies rye bread.
Milk, signifies ½ pint.

TABLE XI.

I. N.—Fermentable and non-fermentable sugar in urine on carbohydrate-rich diet.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 23							
7 a.m.– 9 a.m.....	116	0.047	55.6	35.4	0.017	20.2	36.3
9 " –11 "	58	0.113	65.6	32.2	0.057	33.4	50.9
11 " – 1 p.m.....	81	0.064	51.9	22.2	0.036	29.7	57.1
1 p.m.– 3 "	52	0.125	65.0	29.3	0.068	35.7	55.0
3 " – 5 "	50	0.211	105.5	40.5	0.130	65.0	61.6
5 " – 7 "	62	0.165	102.3	39.1	0.102	63.2	61.8
7 " – 9 "	51	0.176	89.8	39.9	0.097	49.9	55.6
9 " –11.30 p.m.	200	0.052	105.6	66.4	0.019	39.2	37.1
11.30 p.m.–7 a.m...	580	0.035	205.9	120.1	0.014	85.8	41.7
7 a.m.– 7 a.m.....	1,250	0.067	847.2	425.1	0.033	422.1	49.8

Breakfast: Two rolls, butter, cantaloupe, milk, sugar.. 8.00 a.m.
Lunch: Two rolls, cake, ice-cream, orange, peach..... 1.10 p.m.
Supper: Potatoes, cream, bread, butter, milk, ice-cream. 7.20 "

July 25							
7 a.m.– 9 a.m.....	96	0.062	59.6	24.4	0.036	35.2	59.1
9 " –11 "	43	0.200	86.0	31.8	0.126	54.2	63.0
11 " – 1 p.m.....	71	0.128	90.9	35.9	0.077	55.0	60.5
1 p.m.– 3 "	78	0.104	81.1	36.9	0.056	44.2	54.5
3 " – 5 "	62	0.224	138.8	48.3	0.146	90.5	65.2
5 " – 7 "	83	0.191	158.5	51.4	0.129	107.1	67.5
7 " – 9 "	62	0.190	118.0	49.8	0.110	68.2	57.9
9 " –11 "	65	0.208	135.2	44.2	0.140	91.0	67.3
11 " – 7 a.m.....	500	0.057	288.0	124.5	0.032	163.5	56.8
7 a.m.– 7 a.m.....	1,060	0.109	1,156.1	447.2	0.066	708.9	61.3

Breakfast: Cantaloupe, sugar, milk, krumbles, one and one-half rolls, butter..... 8.00 a.m.
Lunch: Two rolls, butter, apple, orange, peach, plum, tomato, ice-cream, strudel..... 1.40 p.m.
Supper: Bread, karo syrup, rice, milk, potatoes, butter, cream, pear, ice-cream..... 7.20 "

TABLE XI—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 26							
7 a.m.– 9 a.m.	225	0.027	61.2	34.6	0.011	26.6	43.4
9 " –11 "	52	0.183	95.2	34.9	0.116	60.3	63.4
11 " – 1 p.m.	84	0.130	109.2	34.1	0.089	75.1	68.8
1 p.m.– 3 "	94	0.103	96.8	42.9	0.057	53.9	55.7
3 " – 5 "	60	0.297	178.2	60.0	0.197	118.2	66.3
5 " – 7 "	76	0.261	198.3	49.3	0.196	149.0	75.1
7 " – 9 "	57	0.297	169.3	47.3	0.214	122.0	72.0
9 " –11 "	57	0.288	164.2	54.8	0.192	109.4	66.7
11 " – 7 a.m.	500	0.091	456.0	220.5	0.047	235.5	51.6
7 a.m.– 7 a.m.	1,205	0.126	1,528.4	578.4	0.078	950.0	62.2

Breakfast: Cantaloupe, sugar, one and one-half rolls, karo syrup, milk, krumbles ..... 8.00 a.m.
Lunch: Two rolls, karo syrup, apple, orange, peach, strudel, tomato, ice-cream ..... 1.45 p.m.
Supper: Bread, karo syrup, potatoes, cream, butter, krumbles, milk, sugar, peach pie, ice-cream ..... 7.10 "

food ingestion on the sugar output is more marked than on the previous diets.

Table XII records experiments with I. N. to test the effect of dextrose ingested on an empty stomach. His tolerance for dextrose is much greater than that of E. O. 20 gm. of dextrose ingested by I. N. on an empty stomach had no effect of any kind on the sugar output, and only one of these experiments is included in the table. With 50 gm. of dextrose (July 16 and 17, Table XII) there is also no appreciable effect on the sugar output or on the ratio of unfermentable to fermentable sugar. An increase in the dextrose ingestion to 60 gm. (July 18 and 20) is followed by a definite rise in the total sugar elimination, and in a sharp fall in *percentage* of unfermentable sugar. The absolute tolerance for dextrose on an empty stomach in the case of I. N. lies therefore between 50 and 60 gm. The figure would have been fixed more exactly, and the question would have been answered as to whether

TABLE XII.

I. N.—Experiments to test the effect of varying amounts of dextrose ingested on an empty stomach. 6 to 8 a.m. is taken as the control period in each experiment. Details of dextrose ingested and subsequent diet are given below.

Date	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 16							
6 a.m.– 8 a.m.	82	0.049	40.2	23.1	0.020	17.1	42.7
8 " –10 "	48	0.082	39.5	15.5	0.050	24.0	60.8
10 " –12 noon	42	0.125	52.5	24.5	0.066	28.0	53.3
12 noon– 2 p.m.	57	0.090	51.4	29.3	0.038	22.1	43.1
2 p.m.– 6 "	580	0.072	419.3	222.7	0.033	196.6	46.9
6 a.m.– 6 a.m.	809	0.074	602.9	315.1	0.035	287.8	47.7
July 17							
6 a.m.– 8 a.m.	121	0.030	36.9	21.3	0.012	15.6	42.3
8 " –10 "	58	0.068	39.9	22.4	0.030	17.5	43.7
10 " –12 noon	60	0.076	46.1	25.8	0.033	20.3	44.0
12 noon– 2 p.m.	82	0.055	45.6	25.1	0.025	20.5	45.0
2 p.m.– 6 "	620	0.072	449.5	216.4	0.037	233.1	51.9
6 a.m.– 6 a.m.	941	0.065	618.0	311.0	0.032	307.0	49.7

July 16

6 a.m. 100 cc. water.
8 " 100 " " + 50 gm. dextrose.
Breakfast: Roll, butter, egg, milk.....10.15 a.m.
Lunch: Two rolls, minster cheese, orange, peach..... 2.10 p.m.
Supper: Bread, butter, cream cheese, onions, milk, malted milk..... 7.20 "

July 17

6 a.m. 100 cc. water.
8 " 100 " " + 50 gm. dextrose.
Breakfast: Roll, butter, egg, milk.....10.00 a.m.
Lunch: Two rolls, Swiss cheese, apple, orange, butter, tomato..... 2.15 p.m.
Supper: Bread, butter, fried fish, two eggs, milk..... 7.00 "

TABLE XII—*Concluded.*

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 18							
6 a.m.– 8 a.m.	120	0.031	37.9	18.9	0.015	19.0	50.0
8 " –10 "	70	0.091	64.1	43.7	0.029	20.4	31.8
10 " –12 noon	34	0.131	44.5	22.7	0.064	21.8	48.9
12 noon– 2 p.m.	65	0.068	44.4	22.1	0.034	22.3	50.2
2 p.m.– 6 "	600	0.077	465.6	281.4	0.030	184.2	39.6
6 a.m.– 6 a.m.	889	0.073	656.5	388.8	0.030	267.7	40.8
July 19							
6 a.m.– 8 a.m.	126	0.030	38.2	19.5	0.014	18.7	48.9
8 " –10 "	76	0.048	37.1	18.1	0.025	19.0	51.2
10 " –12 noon	50	0.099	49.8	24.5	0.050	25.3	50.9
12 noon– 2 p.m.	65	0.077	50.5	22.0	0.043	28.5	56.4
2 p.m.– 6 "	650	0.067	440.6	161.7	0.042	278.9	63.3
6 a.m.– 6 a.m.	967	0.063	616.2	245.8	0.038	370.4	60.1

July 18

6 a.m. 100 cc. water.
8 " 100 " " + 60 gm. dextrose.
Breakfast: Roll, egg, butter, milk.....10.15 a.m.
Lunch: Two rolls, cream cheese, apple, orange, butter, peach..... 2.10 p.m.
Supper: Bread, cutlets, cucumbers, cooked cherries.... 7.05 "

July 19

6 a.m. 100 cc. water.
8 " 100 " " + 20 gm. dextrose.
Breakfast: Roll, egg, butter, milk.....10.10 a.m.
Lunch: Two rolls, butter, Swiss cheese, orange, peach, tomato..... 2.15 p.m.
Supper: Bread, salmon, onions, milk, butter, malted milk..... 6.45 "

July 20							
6 a.m.– 8 a.m.	108	0.041	45.0	18.0	0.025	27.0	59.9
8 " –10 "	68	0.088	60.0	31.6	0.041	28.4	47.3
10 " –12 noon	56	0.125	70.0	37.5	0.058	32.5	46.5
12 noon– 2 p.m.	75	0.095	71.5	36.5	0.046	35.0	49.0
2 p.m.– 6 "	550	0.091	504.4	217.9	0.052	286.5	56.8
6 a.m.– 6 a.m.	857	0.087	750.9	341.5	0.047	409.4	54.5

6 a.m. 100 cc. water.
8 " 100 " " + 60 gm. dextrose.
Breakfast: Roll, butter, egg, milk.....10.00 a.m.
Lunch: Two rolls, butter, cream cheese, apple, orange, tomato..... 2.00 p.m.
Supper: Bread, cutlets, cucumbers, cooked apple..... 7.00 "

I. N. (as E. O.) shows the first tolerance limit for dextrose on an empty stomach in a changed ratio of the two urinary sugars had not the Government draft law prevented completion of the experimental work. Table XIV shows, however, that I. N. may react to dextrose exactly as did E. O., by the changed ratio of the urinary sugars.

Table XIII presents condensed results of experiments with I. N. to determine the effect of adding dextrose to a weighed breakfast. The breakfast contained no sugar, other than a few gm. of lactose in the milk. August 13 and 15 serve as control periods, showing the sugar output after the breakfast eaten at 8 o'clock. On August 14, 16, and 18, dextrose was taken with the breakfast, 20 gm. on the 14th, 40 on the 16th, and 60 on the 18th. As opposed to the results on an empty stomach, it will be noted that even 20 gm. of dextrose with breakfast caused an appreciable increase in the total sugar elimination following the breakfast. This increase is more marked with 40 gm. of dextrose, and increases still further with 60 gm. On this latter diet the total sugar increase (over the breakfast alone) amounts to more than 60 mg. accompanied by a marked fall in percentage of the unfermentable sugar. 20 or 40 gm. of dextrose on an empty stomach produced *no* increased sugar elimination (Table XII) while 60 gm. produced less than half the increase noted in the present experiment. This result, which parallels closely the similar experiments with E. O., serves again to support the view that digestive activity of the pancreas may interfere with its internal function to some extent.

Table XIV presents an experiment on I. N. to determine the effect of addition of 25 gm. of dextrose to each meal of a weighed diet. The first 3 days serve as controls. The plan of experiment is similar to that reported in Table VIII for E. O. In the latter experiment the total sugar elimination was more than doubled. The increased sugar eliminated was chiefly fermentable, so that the percentage of unfermentable sugar fell to a very low figure. Table XIV shows the much higher tolerance which I. N. has as compared with E. O. The addition of the 25 gm. of dextrose to each meal causes no increase in the total sugar elimination. The total quantity of sugar put out for the 3 experimental days varies only within expected limits from the control period. Never-

TABLE XIII.

I. N.—Experiments to test the effect on the urinary sugar output of varying quantities of dextrose added to a weighed breakfast.

The same breakfast was eaten at 8 a.m. each morning. It consisted of:

	gm.
Bread	74
Butter	5
Milk	233
One egg.	

Date	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	cc.	per cent	mg.	mg.	per cent	mg.	per cent
Aug. 13							
a.m.							
8–10	76	0.074	56.3	31.9	0.032	24.4	43.3
10–12	86	0.060	52.1	22.6	0.034	29.5	56.6
Aug. 15							
8–10	124	0.046	57.9	32.1	0.020	25.8	44.5
10–12	80	0.071	57.1	25.3	0.039	31.8	55.6
Aug. 14	(20 gm. dextrose added to breakfast.)						
8–10	106	0.068	72.1	34.3	0.035	37.8	52.5
10–12	70	0.082	57.4	18.5	0.055	38.9	67.7
Aug. 16	(40 gm. dextrose added to breakfast.)						
8–10	100	0.077	77.3	44.8	0.032	32.5	42.0
10–12	84	0.074	62.2	29.6	0.038	32.6	52.4
Aug. 18	(60 gm. dextrose added to breakfast.)						
8–10	94	0.097	91.3	68.9	0.023	22.4	24.5
10–12	64	0.120	76.8	47.7	0.045	29.1	37.9

theless an effect of the sugar addition to the diet is perfectly apparent, in that both absolute quantity and percentage of the unfermentable sugar are markedly decreased. This effect is only slightly apparent on the 1st day, but the 2 following days leave no room for question. This result exactly duplicates that for E. O. reported in Table V, but in the latter case the changed urinary relationship of the sugars followed ingestion of 20 gm.

TABLE XIV.

I. N.—To study the effect of addition of 25 gm. of dextrose to each meal (weighted diet). The first 3 days serve as controls.

Date.	Volume.	Before fermentation.		Fermented.	After fermentation.		
							Non-fermentable.
1917	*cc.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
July 30							
7 a.m.–7 p.m.	455	0.094	431.3	226.9	0.044	204.4	47.4
7 p.m.–5 a.m.	215	0.082	176.7	47.3	0.060	129.4	73.2
5 a.m.–7 "	101	0.039	39.4	11.4	0.027	28.0	71.0
7 a.m.–7 a.m.	771	0.084	647.4	285.6	0.046	361.8	55.9
July 31							
7 a.m.–7 p.m.	385	0.100	385.0	179.8	0.053	205.2	53.3
7 p.m.–5 a.m.	146	0.130	189.8	45.6	0.098	144.2	76.0
5 a.m.–7 "	73	0.056	41.2	13.2	0.038	28.0	68.1
7 a.m.–7 a.m.	604	0.102	616.0	238.6	0.062	377.4	61.2
Aug. 1							
7 a.m.–7 p.m.	355	0.105	372.8	182.2	0.053	190.6	51.1
7 p.m.–5.15 a.m.	174	0.115	200.1	41.4	0.091	158.7	79.3
5.15 a.m.–7 "	62	0.050	31.3	14.9	0.026	16.4	52.5
7 a.m.–7 a.m.	591	0.102	604.2	238.5	0.061	365.7	60.5
Aug. 2							
7 a.m.–7 p.m.	322	0.123	396.1	212.2	0.057	183.9	46.4
7 p.m.–5 a.m.	180	0.111	199.8	55.6	0.080	144.2	72.2
5 a.m.–7 "	79	0.044	35.5	17.1	0.023	18.4	51.9
7 a.m.–7 a.m.	581	0.109	631.4	284.9	0.059	346.5	54.9
Aug. 3							
7 a.m.–7 p.m.	370	0.107	396.0	259.5	0.036	136.5	34.5
7 p.m.–5 a.m.	550	0.037	207.4	82.5	0.022	124.9	60.2
5 a.m.–7 "	70	0.047	33.2	17.0	0.023	16.2	49.0
7 a.m.–7 a.m.	990	0.064	636.6	359.0	0.028	277.6	43.6
Aug. 4							
7 a.m.–7 p.m.	720	0.051	373.7	261.4	0.015	112.3	30.0
7 p.m.–5 a.m.	225	0.090	203.4	112.3	0.040	91.1	44.8
5 a.m.–7 "	64	0.050	32.1	18.7	0.021	13.4	41.8
7 a.m.–7 a.m.	1,009	0.060	609.2	392.4	0.021	216.8	35.6

Diet, July 30, 31, Aug. 1.

Breakfast: 50 gm. corn flakes, 1 pint milk.....485 gm.
Lunch: 60 gm. uneeda biscuits, 1 pint milk.....490 "
Supper: 60 gm. " " 2 eggs.....100 "

Aug. 2, 3, 4, same as above + 25 gm. pure dextrose with each meal.

of glucose on an empty stomach. The limit of tolerance to dextrose is widely different in these two subjects, yet when we reach the border-line effect of the sugar in either one this effect becomes first apparent in a change in the nature of at least a portion of the sugar eliminated.

DISCUSSION.

Tolerance.—The method of determining tolerance for carbohydrate which has been practically universally employed is to feed or inject the sugar to be studied and then test the urine for sugar at indefinite periods by one of the qualitative copper reagents. When a qualitative reaction is observed the limit of tolerance is said to have been reached. The fundamental inaccuracies of such a procedure are obvious.

When used to test a pure sugar solution, Fehling's reagent will give a distinctly recognizable reaction with 0.001 per cent of dextrose. Yet with ordinary urines, Fehling's solution frequently fails to give a definite reaction with 0.1 per cent of dextrose present. Thus where Fehling's solution is employed, it requires about 100 times as much sugar to give a positive reaction in urine as in pure solution. With the Benedict copper test the discrepancy is much less, but is always considerable. The factors lessening the delicacy of the copper tests for sugar in urine are probably numerous. Two of these can be mentioned specifically; *viz.*, creatinine and urea. The former holds cuprous oxide in solution directly, the latter indirectly, through continuous production of traces of ammonia during the heating. Creatinine is the chief interfering substance but urea contributes to some extent to decrease the delicacy of the copper tests when applied to urine. It is plain then that simple variations in the concentration of the interfering substances may lead to erroneous results when testing carbohydrate tolerance by direct qualitative copper tests. Sugar may increase very appreciably in urine before being detected by such a method.

We have shown in this and the preceding paper that urine constantly contains sugar, and that this sugar may undergo variations as wide as 100 per cent or more in absolute amount per hour, and in percentage in the urine. Most of these variations would wholly escape detection by qualitative methods.

The common idea that glycosuria is a sudden phenomenon, indicated by the appearance of a positive sugar test by a copper reagent must be wholly discarded. Sugar elimination takes place continuously through the urine, and an accurate measure of the influence of any substance on sugar excretion can be determined only by a quantitative determination of the sugar content of the urine for a period of at least 2 hours before and after administration of the substance to be tested. The relationship to food ingestion must be kept constant during such an experiment. The periods for examination of the urine should not be much longer than 2 hours, or a significant sugar increase may be lost in the normal fluctuation for a longer time (cf. Myers[13]). Thus an increase of 15 mg. produced in the sugar eliminated for the first 2 hours would usually be definitely positive, while the same absolute increase in the urine of 5 or 6 hours might warrant no conclusion. Since the effect on the sugar excretion may extend over only a short period of time, the accurate detection of such an effect may well depend upon examination of the urine for the corresponding period. Volume of urine and qualitative tests are of little significance; the real criterion is absolute quantity of sugar eliminated per hour. For the very closest study of effect on sugar elimination a further refinement of technique is necessary. The point will be discussed below.

Our studies on both man and the dog have shown that there is no absolute tolerance for carbohydrate in either of these species except during the fasting state. We shall report in detail upon sugar tolerance in the dog in a later paper.

In man there is apparently a real tolerance for glucose and for cane sugar, during fasting. This tolerance is much lower than is generally supposed, and varies widely with different individuals. One of our subjects showed an effect of 20 gm. of dextrose on an empty stomach, the other showed the first effect after ingesting 60 gm. of dextrose on an empty stomach. From present limited studies of other individuals, we are inclined to look upon 50 gm. of glucose as about the upper limit of absolute tolerance in normal human adults. This tolerance may be manifest only during fasting. Feeding with either meat or a carbohydrate diet causes an increase in the sugar elimination.

Glycosuria.—Progress in the study of carbohydrate metabolism will probably be more rapid if the term "glycosuria" can be abolished. This word was not created by the mind of man, but by the inefficiency of the copper tests. Glycosuria implies a sudden point at which sugar appears in the urine. Since there is no such point, the term glycosuria is without special significance and is misleading.

A term is needed which includes both the constant presence of sugar in normal urine, and an excess of sugar elimination above the normal. Such a term would serve as a reminder to the student of the subject that the normal urine contains sugar, that this sugar is subject to change through various influences, and that it is worthy of some attention. Possibly "glycuresis"[14] would be a satisfactory term, as indicating *increase* of sugar, not a new appearance of sugar, in the urine. The word is unobjectionable from the point of view of derivation. It lends itself readily to the formation of derivatives; *e.g.*, glycuretics, substances which increase sugar in the urine (phlorhizin, adrenalin, etc.). A simple term for this class of substances has hitherto been wanting.

As a matter of convenience we shall adopt the term glycuresis in the succeeding portions of this paper. It signifies an increase in the urinary sugar elimination above that of the control period. By increase we never refer to increase of percentage of sugar in the urine (except when so specified) but to increase in the absolute elimination of sugar per hour.

Influence of Food.—Careful investigations on the influence of food ingestion on the urinary sugar elimination have not hitherto been reported. Schöndorff[5] stated that increase of carbohydrate in the diet increased the percentage of sugar in the urine, but did not investigate the question in greater detail. Our results show a definite glycuresis for both man and dog following the ingestion of food, even when such food is wholly free from sugar. As previously mentioned, this finding supports the view that the internal function of the pancreas suffers during the exercise of its digestive function. Cohnheim and Klee[15] reached a similar con-

[14] "Glucuresis" might seem preferable to some.

[15] Cohnheim, O., and Klee, P., *Z. physiol. Chem.*, 1912, lxxviii, 464.

clusion through work with dogs having a pancreatic fistula. These authors reported that foods producing the least flow of pancreatic juice have least tendency to cause sugar elimination. They found that oat meal produces least flow of pancreatic juice, and adopt the above view in explanation of the results with the so called "oat cure" in diabetes. Allen tends to accept the same view.

The fact that foods, even when free from any sugar, cause marked glycuresis must serve to emphasize the narrow margin of absolute sugar tolerance even in the normal individual. A certain portion of the sugar from starch or even from meat taken at each meal is waste sugar. This is not due to simple leakage through the kidney. The glycuresis is independent of urinary volume and follows a definite course, reaching a minimum during the fasting hours. Carbohydrate stands apart from other foodstuffs in that the normal organism fails to utilize it completely even in physiological quantities. The unique disease, diabetes, springs from seed ever present in the normal organism.[16]

The Assimilation Limit for Dextrose.—Results on both of our subjects have shown that the assimilation limit for dextrose is not identical with the threshold of glycuresis. The first detectable effect of dextrose ingestion may be a changed ratio of fermentable to unfermentable sugar in the urine, even where the absolute amount of total sugar eliminated is not increased.

We feel that it would not be profitable at the present time to attempt any theoretical discussion of the questions involved in this finding. Further data are necessary on the behavior of the two sugars, including studies on the influence of starch as compared with sugar, and the behavior of the non-fermentable sugar in various forms of glycuresis, including severe diabetes.

[16] It is correct to say that the normal organism is truly diabetic, if we accept the common view of diabetes as a disease in which there is partial or complete failure in utilization of carbohydrate. Allen's criteria of diabetes are: (1) that dextrose in the normal individual behaves as an antidiuretic while in the diabetic it acts as a diuretic, and (2) that in the non-diabetic organism (even where there is glycuresis (glycosuria)) the more dextrose that is given, the greater will be the absolute quantity utilized. Since neither of these criteria hold in mild cases of diabetes mellitus, or in partially depancreatized dogs, the definition cannot be accepted except for "complete" diabetes; *i.e.*, where there is practically no utilization of sugar whatever.

We shall therefore reserve any discussion for future communications.

Dextrose in Its Relation to Glycuresis.—There has been much discussion of the possible etiological relationship of sugar ingestion to diabetes, but definite conclusions have not been reached. We feel that some of the data in the present paper may bear on the question. It will be noted that in E. O. (Table V) 20 gm. of dextrose on an empty stomach cause no glycuresis whatever. Yet the same quantity of dextrose added to a weighed breakfast (Table VI) increases the sugar output by over 250 mg.

The 2 last experimental days reported in Table VIII appear to represent a real breakdown in carbohydrate metabolism. Yet these days differ from the 3 preceding only in the addition of 25 gm. of dextrose to each meal. The effect of this simple addition of dextrose *to the meals* is so marked that the subject (who was analyzing his own urine) preferred not to continue the experiment through the 3rd day. Indeed the picture in Table VIII is such that no one would probably feel justified in urging any prolonged continuation of the experiment. I. N. had a much higher tolerance than E. O., but Table XIII shows that 20 gm. of dextrose added to breakfast caused glycuresis, while 20 or even 40 gm. on an empty stomach have no such effect.

A further subject, not reported in detail in the present paper, is of interest in this connection. He is a young medical student of vigorous appearance, with a family history of diabetes. One grandparent died of the disease, and both parents showed traces of sugar at life insurance examination, but neither has developed diabetes. This subject upon an ordinary mixed diet showed usually a sugar elimination considerably under 1 gm. per day, but upon occasional days this elimination was increased to 2 gm. and over. When traced out it was found that these sugar outpourings occurred on days when apple cake was eaten. A sample of the cake was secured and analyzed. It contained over 12 per cent reducing sugar which was nearly all fermentable and which was presumably commercial dextrose.

Thus our results seem to show unmistakably that dextrose taken with a meal may constitute a strain on the carbohydrate function. Even in a strictly normal individual, as E. O., the strain may be so clear cut as to warrant caution in the use of

dextrose with meals. The rapid absorption of dextrose into the blood stream during digestion may apparently overthrow the carbohydrate balance in man in remarkable fashion. There is no evidence that such effect would be permanent even if long continued, but we scarcely care to attempt such an experiment.

Some Clinical and Therapeutic Considerations.—Although our work has been carried out wholly on normal individuals, there are certain aspects which may be of clinical or therapeutic interest. With the exception of the first point mentioned below, they are cited as chiefly, if not wholly, of theoretic interest at the present time.

In attempting to diagnose or treat the early stages of lowered sugar tolerance the clinician has had very definite difficulties. He has had to rely wholly upon qualitative tests which necessarily fail to afford definite data on the question he is studying. The results by such technique upon individual samples of urine may be entirely misleading, and may fail to demonstrate any abnormality in a 24 hour sample of urine, though such urine may contain 50 per cent more sugar than it should.

The question can now be at least placed upon a more definite basis. Where there is any suspicion of an abnormally low sugar tolerance an occasional analysis should be made of the 24 hour sample of urine (on the ordinary diet) by the method detailed in the first of the accompanying papers. If the total sugar elimination amounts to more than 1.5 gm. per day the diet should be altered until this figure is reached as the upper limit. Further work may lead to revision of the figure 1.5 as maximal for an ordinary diet in an adult, but we feel reasonably sure that if such revision comes it will be downward.

Our results have shown an increased sugar elimination following each meal, irrespective of the nature of the food eaten. Mere digestion and absorption of food lead to glycuresis. This finding naturally suggests that the diabetic might profitably eat his total food intake in one or in two meals instead of the ordinary three. Whether improved assimilation would repay such a trial is problematical. We freely admit that further work is necessary to give the point sound theoretical basis.

It was pointed out earlier in this paper that ingestion of a small quantity (20 gm.) of dextrose in 100 cc. of water before breakfast